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COMPARATIVE CHEMOTHERAPY OF SYSTEMIC VACCINIA IN RABBITS

DAVID STANLEY FEDSON

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COMPARATIVE CHEMOTHERAPY OF
SYSTEMIC VACCINIA IN RABBITS

A thesis presented to the Faculty of the
Yale University School of Medicine in partial fulfillment
of the requirements for the degree
Doctor of Medicine

David Stanley Fedson
B.A., Yale University, 1959

The Department of Medicine
The Department of Epidemiology and Public Health
Yale University School of Medicine
New Haven, Connecticut

May, 1965

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Finally, I would like to express my gratitude to the students of the Yale School of Medicine who support the Peter Parker Fellowship, and to the Ella Lyman Cabot Trust. Their generosity enabled me to visit India where I saw smallpox for the first time. This experience underlies all that is in the following pages.

"Thus far have I proceeded in an inquiry founded, as it must appear, on the basis of experiment; in which, however, conjecture has been occasionally admitted in order to present to persons well situated for such discussions objects for a more minute investigation. In the meantime I shall continue to prosecute this inquiry, encouraged by the hope of its becoming essentially beneficial to mankind."

Edward Jenner

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I. INTRODUCTION

During the summer of 1964 the author was afforded the opportunity to travel and study in India, and it was in Madras at the Infectious Diseases Hospital that he saw his first cases of smallpox. Although little in the way of therapy could be offered the patients, a study in this hospital a few years earlier had demonstrated that a compound called N-methyl-isatin-B-thiosemicarbazone was of prophylactic benefit in smallpox but was ineffective once clinical disease had appeared (26).

The Department of Pharmacology at the Yale School of Medicine has for many years been engaged in the search for effective antagonists to the biosynthesis of nucleic acids. The goal has been to develop agents which might be useful in the treatment of neoplastic disease. One of the most exciting developments arising from these studies has been the demonstration that some of these compounds possess antiviral activity against viruses containing deoxyribonucleic acid (DNA). Among these 5-iodo-2'-deoxyuridine and 6-azauridine have been particularly promising, and it has been suggested that they might be useful in the treatment of severe DNA-virus infections such as smallpox.

This thesis reports an experiment in which a severe systemic poxvirus infection was created in rabbits with vaccinia, a virus closely related to smallpox. An attempt has been made to compare the antiviral activity of N-methyl-isatin-B-thiosemicarbazone, 5-iodo-2'-deoxyuridine and 6-azauridine in this system.

As part of this study, a review of the pathogenesis of poxvirus infections and previous experience in the chemotherapy of viral infections with these three agents has been undertaken. The mechanisms of viral infections have been discussed in a number of general reviews (13, 19, 57, 58, 130, 171, 184, 240, 254, 262). More specific information on the group of poxviruses has also appeared (75, 91, 92). Summarys of developments in the chemotherapy of neoplastic disease contain much that is relevant to the field of antiviral chemotherapy (47, 145). Progress in antiviral chemotherapy itself has been examined by many authors (222, 242, 244, 245, 246). Familiarity with the broad range of problems in poxvirus infections and previous experience in antiviral chemotherapy may contribute to an understanding of the system that has been employed in this experiment. It forms a background against which the results may be evaluated and criticized. More importantly, it is hoped that such knowledge will suggest further studies which may contribute to the continuing search for an effective agent against clinical smallpox.

As part of this study, a review of the literature of [illegible] infections and personal experience in the epidemiology of [illegible] tions with other forms appears has been considered. The [illegible] viral infections have been discussed by a number of authors, [illegible] (13, 15, 27, 55, 154, 171, 185, 191, 196, 227). Some specific [illegible] mation on the group of [illegible] has also appeared ([illegible]. [illegible] Summary of observations in the epidemiology of [illegible] contains much that is relevant to the field of [illegible] (47, 149). Progress in antiviral chemotherapy itself has been [illegible] by many authors (125, 141, 200, 212, 240). The [illegible] nature of problems in [illegible] infections and [illegible] antiviral chemotherapy may contribute to an understanding of the [illegible] that has been reported in this [illegible] against which the results can be evaluated and [illegible] accurately, it is hoped that this [illegible] which may contribute to the [illegible] toward [illegible].

II. THE PATHOGENESIS OF POXVIRUS INFECTIONS

Poxvirus Infections in Animal Hosts

Early observations

The occurrence of the generalized cutaneous eruption soon after the onset of symptoms in smallpox early led to the assumption, so obvious at first glance, that the disease was one of the epidermis. Thinking of this sort hampered experimental efforts to understand the nature of similar poxvirus diseases in animals. Even when virus had been inoculated intravenously rather than through the usual cutaneous route, attention was directed to lesions in the skin, mucous membranes and genitalia. It was not until 1929 that a proper understanding of the pathogenesis of poxvirus infection began to take form.

The production of lesions in the visceral organs as a result of poxvirus infection had received scant attention before Douglas, Smith and Price made their important observations (73). In preliminary studies only a few lesions had been seen in the lungs and liver and these were neither widespread nor severe. It was not until rabbits were inoculated intratesticularly with vaccinia that significant disease appeared. As they described it, "the most constant and striking feature at autopsy, apart from the condition of the testes themselves, was the presence of a clear, straw-colored peritoneal exudate of semigelatinous consistency which set to a firm clot a few

minutes after withdrawal; occasionally it was turbid and tinged with blood." Previous studies with neurovaccinia had been reported to be inocuous, but taking this testicular strain of vaccinia they proceeded to inoculate rabbits intraperitoneally and produced a fatal infection. At autopsy the peritoneum was congested, showing punctate hemorrhages. The abdominal cavity contained a semigelatinous fluid, and there were numerous fibrous adhesions. The liver was sprinkled with "small round white spots"; the spleen likewise. The adrenals were soft and friable, the omentum "rolled up into a sausage shaped mass." There was intense hemorrhagic congestion of the uterus and bladder, and the lungs showed "glistening greyish nodules with a hemorrhagic spot in the centre."

The demonstration of severe internal involvement together with later isolation of virus clearly demonstrated the generalized nature of this poxvirus infection. No speculations were offered as to the pathways of infection or the types of cells involved. It remained for Ledingham to deliver the final blow to the notion that vaccinia had a specific "epidermotropism" (159). In studies on the histology of vaccinial skin lesions in rabbits, after either subcutaneous, intraperitoneal or intrasplenic inoculation, he found the virus had little affinity for "epiblastic" elements. In all cases the skin lesions were lesions of the local reticulo-endothelial system. When India ink was inoculated locally, either prior to or at the time of infection with vaccinia, Ledingham observed hyperplasia of local reticulo-endothelial elements (160). No skin lesions were produced, even when the virus was

inoculated as much as 49 days later. India ink was ineffective when given intravenously, and by itself did not kill the virus.

These important observations on the role of the reticulo-endothelial system in vaccinia infection were later extended by Beard and Rous (28). They found that Kupffer cells isolated *in vitro* and then mixed with vaccinia, would, on inoculation in the rabbit skin, cause suppression of a normal vaccinial lesion. This suppression was not due to toxic products of autolysed transferred cells, nor to any circulating factor in the serum of the host. At the same time, the apparently paradoxical observation was made that Kupffer cells inoculated with vaccinia and maintained *in vitro* supported rather than suppressed viral growth, the cells remaining in good condition. In both cases, the Kupffer cells were obtained from animals not previously sensitized to vaccinia and the authors offered no explanation for their findings.

An initial understanding of the pathways of established poxvirus infection grew out of the work of Yoffey and Sullivan (264). They administered vaccinia intranasally to rabbits and at timed intervals collected samples from the cervical and thoracic lymph ducts, blood and from cervical and mesenteric lymph nodes. Virus was found in the cervical lymph 12 hours after infection. It was shown to pass through lymph nodes and appeared in the thoracic duct in small concentrations. Of great importance was their confirmation of Smith's earlier observation that virus was fixed by circulating white blood cells (239). Yoffey found vaccinia fixed to lymphocytes and in this state the virus

was protected from neutralization by antisera. Fairbrother's previous and conflicting findings (85) that circulating white blood cells plus antisera could be more destructive to virus than antisera alone left open the question of the specific role of lymphocytes, but Yoffey and Sullivan suggested that the ability of virus to pass through lymph nodes might be the result of their association with lymphocytes, and that the perivascular accumulation of lymphocytes in the later lesions of vaccinia infection might reflect the cells having carried the virus to these sites.

Rabbitpox, ectromelia and a theory of pathogenesis

While early progress in the elucidation of the pathogenesis of poxvirus infections was made with vaccinia, subsequent advances have been made largely through the study of two closely related viruses, rabbitpox and ectromelia. Rabbitpox first appeared at the Rockefeller Institute in 1932, apparently as an offshoot of a strain of neurovaccinia (112, 113). This virus, and to an even greater extent the Utrecht strain isolated later, is highly virulent for rabbits. There is a short incubation period of from three to six days before the onset of anorexia, weight loss, malaise, diarrhea and fever. A purulent discharge appears in the eyes and nose and pock-like lesions rapidly appear, first at the muco-cutaneous junctions and later in the skin, providing the animal lives long enough. A rash usually appears on the sixth day; and death if it occurs comes 7 to 12 days after inoculation. Lesions are found in many internal organs, especially the lungs, liver and spleen. These was protected from [illegible] in [illegible]. [illegible] questions are [illegible]. [illegible] (19[illegible]) gave interesting [illegible] from [illegible] could be more [illegible] than [illegible] with the [illegible] of the [illegible] in [illegible]. [illegible] suggested that the ability of [illegible] to [illegible] arose early in the [illegible] of [illegible] and that the [illegible] of [illegible] in [illegible] of vascular [illegible] before [illegible] (Green 19[illegible]).

[illegible]

While early progress in the elucidation of the mechanisms of [illegible] was made largely through the [illegible] of [illegible] [illegible] in 19[illegible] [illegible] diffuse nodular lesions are often found in intimate relation to blood vessels.

Ectromelia became the object of extensive studies by Fenner in the late 1940's (86-90). Burnet had first suggested the close relationship between ectromelia and vaccinia by showing that ectromelia would agglutinate the same range of chicken red blood cells as vaccinia, while in addition agglutinating mouse cells (40). Fenner proceeded to show that ectromelia would easily infect the chick embryo and mouse, but not rabbits, rats or guinea pigs.

In the mouse, ectromelia produced a generalized disease, involving the skin, lymph nodes, spleen, liver and many other organs. Though it has recently been questioned (35), Fenner thought that natural disease occurred after the virus entered through abrasions in the skin. Within 7 to 8 days a primary lesion developed at this site. Mice either died within the next four days or went on to develop a generalized rash. Animals dying before the appearance of the rash nevertheless had virus in their skin. Virus could be recovered from livers and spleens of all animals. The livers showed gross as well as microscopic foci of necrosis, randomly distributed. In severe infection these foci were semiconfluent, and there was a considerable increase in the fat content of these organs. There were only small accumulations of lymphocytes around portal triads, with fewer cells scattered throughout the parenchyma. Animals that survived showed no residual fibrosis. In the spleen virus appeared one day earlier than in the liver and lesions were generally

more severe, with fibrosis often following recovery.

In further experimental studies Fenner inoculated mouse foot pads with small doses of the highly virulent Moscow strain of ectromelia, and constructed growth curves for the virus in foot, spleen, blood and skin. Analysis of his data led to a general interpretation of the pathogenesis of mousepox, which he then extended to other acute exanthems (89). Following introduction into the skin, infective virus established itself in regional lymph nodes. After a few days of multiplication necrosis of host cells led to liberation of the virus into the blood. Virus from this primary viremia was taken up by phagocytes in the spleen, liver and possibly bone marrow. Infection and multiplication of virus in these and contiguous cells led again to necrosis, setting up a secondary viremia. This was evident four days after infection. With dissemination virus could be recovered from the skin after six days. The primary lesion in the skin was evident at about this time, signifying an end to the incubation period. Generalized skin lesions appeared only if viral titers in the liver and spleen were not such that the animal died before sufficient multiplication had taken place in the skin. With the onset of skin lesions, the animals became infective and remained so until the scabs had fallen off, usually after 18 days. In animals that recovered, virus concentrations in the spleen began to fall on day 10, and virus disappeared from the blood on day 12. This correlated closely with the rise in circulating antibody, as measured by anti-hemagglutinin titers.

Fenner's studies and hypothesis served to explain the long incubation

period consequent to a two-stage viremia and the onset of symptoms which was due to necrosis in the primary focus, the visceral organs, prior to involvement of the secondary focus, the skin. Fenner argued that multiplication of virus itself, rather than "allergy" was responsible for the cutaneous lesions. In addition, the shorter incubation period of experimental infections produced by either intravenous or intraperitoneal inoculation could be explained by the bypassing of the initial resistances offered by regional lymph nodes prior to primary viremia (88, 90).

The macrophage and virus infection

Up to this time, the pathogenesis of poxvirus infection had been studied primarily by examining tissues grossly and histologically and by taking tissue samples at varying times during the infected period and assaying them for virus. Fruitful as these techniques had been, they could offer no direct proof as to the site of initiation of infection within a particular organ, its spread within that organ and the particular cells which supported virus growth. It is only within the last decade that these problems have been studied with the aid of newer fluorescent antibody techniques, and many of these questions have now been answered.

The outstanding work of Mims has clarified many of these problems in poxvirus infections, and has recently been summarized in an exceptional review (184). Largely because of his efforts, the pivotal role of the macrophage has been elucidated. The importance of the reticulo-endothelial system in clearing the blood of foreign particles has been known for some

time, and extensive studies have appeared on the clearance of a variety of foreign particles and bacteria. There have been few studies, however, on the clearance of virus particles from the blood (58, 181). Mims has shown that ectromelia injected intravenously in mice is cleared from the blood by the Kupffer cells of the liver. The virus half-life in blood is 3 minutes. A small proportion of virus remains uncleared and this may be due to the association of the virus with white blood cells and platelets, for this fraction is not cleared when transferred to another animal. With Rift Valley Fever virus the situation is somewhat different; transfer of the uncleared portion results in prompt clearance of all except a similar small fraction. In this case there may be an equilibrium between blood and macrophage, with small amounts of virus being released into the blood.

Virus and macrophage may interact in a number of ways. There may be no uptake of virus as with poliovirus type I, or virus may be taken up and completely destroyed by the macrophage. The latter seems to be the case with the mild infections produced by the CL strain of vaccinia in mice and ectromelia in the rat (184). Normally infective virus neutralized by antibody is also handled in this manner. With others such as influenza and Rift Valley Fever in mice, virus may be taken up by macrophages and transferred immediately to adjacent hepatic cells. With the poxviruses still another mechanism exists. Here ectromelia, and to a lesser extent rabbitpox, are taken up and multiply within macrophages before spreading into hepatic cells. During the first hour after injection

of large doses of ectromelia there is a marked decrease in the amount of viral antigen in Kupffer cells, as demonstrated by fluorescent antibody. Later the amount of antigen increases, neighboring parenchymal cells are soon infected, and within 26-30 hours the mice are dead (182). Only a small proportion of virus is found in the blood, bile or hepatic lymph glands. Individual macrophages may vary in their ability to take up virus, and Mims has calculated that only 1 in 4 macrophages take up ectromelia (184). This is a matter of some significance, for both inherited and acquired differences in susceptibility and resistance to virus infection may operate on the macrophage level (108, 182, 215, 216).

Little is known of the factors that may modify the response of macrophages to virus infection. In mice given endotoxin, for example, there is an initial increase in susceptibility followed by a more prolonged increase in the resistance to infection by gram negative bacteria. With large doses of ectromelia in mice there is a similar effect, but surprisingly, smaller doses of ectromelia cause an increase in mortality after stimulation with endotoxin (106). Rabbits are especially sensitive to endotoxin, and similar studies in this animal would be helpful. Studies using viruses not of the poxvirus group have shown that thorotrast blockade of the reticulo-endothelial system may slow the clearance of virus, while specific antibody may accelerate it. Whether the latter is due to an increased uptake of antibody-coated virus or to an inhibition of the release of a certain fraction of virus normally in equilibrium with the blood is not known (38).

Mims has also studied the response of the spleen to ectromelia (184). Although it is not a vital organ, its role in antibody production, the

tendency for circulating lymphocytes and mononuclear cells to localize there, and its own macrophage system suggest its importance in viral infections. A large intravenous dose of the Moscow strain of ectromelia is first taken up by macrophages lining the sinusoids of the red pulp. Virus grows predominantly in perifollicular regions and within 24 hours spreads to the centers of the follicles. There is less involvement of the red pulp. Splenic foci enlarge more rapidly than foci in the liver. Virus injected subcutaneously reaches the spleen two days after infection, probably cell bound rather than free. The virus then multiplies in much the same way as after intravenous inoculation, but interestingly there is considerably less involvement of the liver. Of special interest are the occasional follicles which are free of virus antigen and possess instead large amounts of RNA, perhaps an indication of antibody synthesis.

Roberts has studied subcutaneous infection of mice with ectromelia (214). With fluorescent antibody he has demonstrated that macrophages take up virus, support its growth and probably carry it deeper into the dermis. Although the Malpighian layer of the epidermis may be infected, the dermis is far more important, infection progressing much more rapidly in this layer. Using tritiated thymidine he has demonstrated dermal hyperplasia in a ring immediately beyond the advancing margin of the lesion. The local edema Roberts attributes to cutaneous anaphylaxis, though it is not clear how this develops. It is equally conceivable that virus infection of the littoral cells of local capillaries which have phagocytising capabilities may lead to a breakdown in local vascular

integrity. This is no small point, for it may be precisely by this mechanism that generalized skin lesions appear and, indeed, the loss of vascular integrity may play a large role in the death of animals, as will be discussed later. It is assumed that regional lymph nodes play a role in the resistance of the host, but with a virulent virus they might supply a ready site for multiplication and actually aid in the dissemination of the virus. Hematogenous spread must also be important after subcutaneous infection for in both mousepox and rabbitpox splenic foci can often be demonstrated before all nodes in the line of infection are involved (30, 184).

Respiratory infection

In respiratory infections with ectromelia in mice Roberts has shown that virus passing beyond the ciliated regions of the respiratory bronchioles is taken up by alveolar macrophages and may grow in these cells (213). Mucosal cells are also infected. Within 3 days the alveolar septa are involved and virus may spread via capillaries. The simultaneous involvement of pulmonary nodes and the spleen, however, suggest that movement of infected macrophages within perivascular, peribronchial and pleural lymphatics might be equally important, as suggested by Yoffey (264). These lymphatic passages are severely dilated and congested at 6 days, when antibody production begins. Death, when it occurs, comes 1 to 3 days later.

Further information on the role of the lymphatic system in respiratory poxvirus infection has been provided by Bedson and Duckworth (30). Using the highly virulent Utrecht strain of rabbitpox in large doses intranasally, they were able to demonstrate early extensive multiplication

of virus in the respiratory tract but interestingly, and reminiscent of Fenner's work in mousepox (88-90), rabbits did not become infectious until day 4 or55, when nasal and conjunctival discharge was present. Early virus multiplication may have taken place in deeper rather than superficial tissues of the respiratory tract but fluorescent antibody studies which could settle this question were not undertaken. Within 36 hours virus was isolated from deep cervical nodes. At two days it was found in the spleen. Wider dissemination into the blood and other tissues was apparent the following day. Virus could be isolated only from the buffy coat of the blood, and was not found before the spleen was involved. Fever usually appeared on the second day and it was noted at this time that virus was contained primarily in the respiratory tract, cervical nodes and in small amounts in the spleen. Nasal and conjunctival discharge and a generalized rash usually occurred 2 to 3 days after the initial fever and were fulminant on the fifth day, with rapidly rising titers of virus in the spleen and liver. Weakness and anorexia were marked, and with the appearance of diarrhea and a decline in fever the rabbits usually died from 8 to 12 days after infection.

In discussing their results, Bedson and Duckworth pointed out their similarity to those of Fenner in his studies on mousepox. Their results do not clearly establish their suggestion that lymphatics are more important than blood vessels as channels for the spread of virus and that the lymph nodes do not serve as barriers to infection. Very large doses of virus were used to infect their animals, and nodal barriers could

have simply been overwhelmed. Also, the spread of virus via pulmonary capillaries into the blood stream, while discounted by these authors and by Roberts, has not been convincingly ruled out. Both routes might have important roles to play. Regarding the mechanism of fever, they suggested that multiplication of virus in the respiratory tract and local lymph nodes beyond the capacity of the spleen and other reticulo-endothelial tissue to remove it from the blood was an important factor. However, Mims' demonstration that the ability of the liver to clear ectromelia was maintained until the final stages of the disease (181) and the more recent studies of Atkins, Cronin and Isacson on the role circulating leucocytes might play in the pathogenesis of fever in virus infections would suggest that other factors are involved (9). Mims has induced peritoneal exudates in mice that were composed of 75% polymorphonuclear leucocytes and demonstrated with fluorescent antibody that after two hours ectromelia was extensively taken up by some but not all of these cells, and that later all of the cells had lysed without any evidence that virus had multiplied (184). The knowledge that polymorphonuclear leucocytes are the major if not the only source of endogenous pyrogen suggests their involvement in poxvirus fever, but the problem has not been studied.

Cerebral and peritoneal infection

The major understanding of the pathogenesis of poxvirus infections has come from studies in which virus was introduced into the skin, the lungs or directly into the circulation. Other sites of inoculation have

have simply been disregarded. Also, the concept of [illegible] equilibria with the [illegible] by Roberts, has not been [illegible] were important roles to play. [illegible] suggested that calcification [illegible] tissue to remove it from the [illegible] demonstration that the [illegible] was maintained until the [illegible] recent studies of Anitschkow, [illegible] leucocytes might play in the [illegible] would suggest that [illegible] leucocytes and [illegible] human [illegible] cells, and that [illegible] that were not [illegible] leucocytes [illegible] the lesion.

[illegible]

The major [illegible] was [illegible] lungs of [illegible] often been used to study other aspects of infection. However, much less is known about the pathogenesis of infection in such sites as the brain and peritoneal cavity. The need for understanding here is of great importance, for these routes of infection are often used in chemotherapy studies. It is Mims again who has contributed to our meager understanding in this area. Intracerebrally injected ectromelia, rabbitpox and several strains of vaccinia have all been shown to grow in the meninges and in cells lining the cerebrospinal fluid spaces (183, 184). There is little if any growth in the parenchyma of the brain itself. Yet the small amounts of virus recovered may be derived from the blood stream, for it has been shown that with intracerebral injections, most of the virus spills over into the blood (183).

In infections of the peritoneal cavity some intriguing data have been gathered (184). The normal cellular population of the peritoneal cavity in mice is made up of 25% macrophages, 65% lymphocytes, and 10% intermediate forms. The macrophages are found free and on the serosal surfaces. When ectromelia is introduced virus can be demonstrated in the blood within a few minutes. The virus is taken up and multiplies in peritoneal macrophages and no doubt enters the subdiaphragmatic lymphatics. Fluorescent antibody studies reveal foci of infection in retrosternal nodes after eight hours. After two days there is massive destruction of peritoneal macrophages. Of great importance is the finding that the liver and spleen are not infected via direct spread from the peritoneal cavity; in fact they are infected later than when virus is given

intravenously. Yet intraperitoneal infection is far and away the most lethal of ectromelia infections in mice; a dose barely infectious subcutaneously routinely causes death (267). The reasons for this are quite unknown. Secondary microbial invasion is a possibility, but Mims has found bacteremia to be uncommon, and tetracyclines do not reduce mortality. While this does not rule out secondary bacterial infection, it seems unlikely. There is the possibility that peritoneal macrophages may be different in some respects from other body macrophages. In severe infections via other routes macrophages and lymphocytes are destroyed in all sites, with the exception of the peritoneal cavity. These cells may be bypassed by infection, or as Mims has speculated, they may serve as a source of supply for systemic macrophages.

Despite those important studies, major questions concerning intraperitoneal infection remain to be investigated; the cellular response to virulent and avirulent viruses, the possibility of factors associated with virulent viruses which inhibit host defense mechanisms, the role of the "toxic" effects of virus, the contributions of immunity, the possibility of endotoxin sensitization, and even the role of the tonicity of the medium in which the injected virus is suspended. These questions have been explored in mice infected with staphylococci (59-61) and similar studies with viruses would be of great interest.

Summary

To summarize briefly, the pathogenesis of poxvirus infection in animals has been studied in mousepox and rabbitpox, and to a lesser

extent with vaccinia. The temporal course of these infections has suggested that a primary and secondary viremia are characteristic. There is disagreement on whether regional lymph nodes act as barriers to infection or facilitate its spread. Virus appears to be primarily associated with circulating leucocytes, but again their role is poorly defined. Hematogenous spread may be of primary or secondary importance.

The involvement of internal organs, particularly those of the reticulo-endothelial system, is well established. Whether the outcome of infection will be recovery, or clinical illness with its toxicity, cutaneous lesions and death, may in large part be determined by the nature of the virus-macrophage relationship. The factors specifically viral or cellular which determine the outcome of their encounter will be among the major considerations in the remaining sections on the pathogenesis of poxvirus infection.

Poxvirus Infection in Cells

Adsorption and phagocytosis

The initial stage of poxvirus infection, the adsorption of the virus particle to host cells, has been one of the least well understood phases of its total cycle of replication. Unlike myxoviruses, little is known of the role of cell receptors, specific or non-specific, so nothing can be said except to point out the lack of knowledge in this area.

Animal viruses do not possess the syringe-like mechanism of a

bacteriophage which injects its nucleic acid into its host cell. For infection by animal viruses to occur, it has been thought the host cell plays an active role in this process and the term "viropexis" was introduced to describe a process that has only recently been elucidated (57). The work of Dales and Kajioka has beautifully demonstrated that vaccinia virus is phagocytized by the host cell (67, 68, Fig. 1). In a series of electron microscopic studies of vaccinia infection in Earle's strain L cells, vaccinia was shown to be engulfed by the cell membrane within a few minutes of adsorption. To determine whether surface-attached virus could inject its nucleic acid without being phagocytized, sodium fluoride was used to inhibit phagocytosis. Fluoride had no effect on adsorption but penetration of the virus was markedly decreased. When fluoride was later removed, phagocytosis resumed, and the membrane-engulfed virus particles were transferred into the cell cytoplasm.

Vaccinia incubated with neutralizing antibody is not infective. When virus is adsorbed to cells immediately prior to incubation at 37°C with antibody it rapidly becomes resistant to neutralization. Adsorbed at 4°C it remains neutralizable, suggesting that the rapid resistance to neutralization at 37°C is dependent upon some function of the cell (201). This resistance is most probably due to the rapid phagocytosis of virus by the cell, separating it from extracellular antibody. However antibody does not prevent the phagocytosis of antibody-coated virus (68). Virus adsorbed to fluoride treated cells is still susceptible to neutralization by antibody. Once fluoride is removed, neutralized virus

is phagocytized. Heat inactivated virus is also taken up.

Source of viral DNA

It has long been known that infection with vaccinia produces characteristic cytoplasmic inclusions. Electron microscopic (102) and fluorescent antibody studies (189) left unsettled the question of the source of viral DNA (220). Microradioautographic studies by Sheek and Magee have provided the answer. Control cultures of Hela cells take up tritiated thymidine (TdR-H^3) in the nucleus; infected cells are labeled in the cytoplasm (174, 177). On infection cells prelabeled with TdR-H^3 do not transfer their nuclear label to the cytoplasm, demonstrating that host cell DNA is not incorporated into new virus (233). Using 5-fluoro-2'-deoxyuridine (FUdR), an inhibitor of thymidylic acid (TdR) biosynthesis, but not its incorporation into DNA, Salzman has demonstrated an inhibition of vaccinia multiplication in Hela cells, thus proving the need for *de novo* synthesis of TdR (224). The amounts present in the cell are insufficient and viral DNA can be formed only from newly synthesized pyrimidines.

Reactivation

Once inside the host cell, the vaccinia particle passes through a latent period before its cycle of replication is complete. During the early stages viral integrity is lost. Studies on chick embryo monolayers (202) and in a number of tissue culture systems (43, 78, 100) have clearly shown that vaccinia enters a true eclipse phase; for a period of several hours after infection virus cannot be recovered from cells which ultimately

produce it. The virus particle is broken down to its various components. It is also clear that initiation of infection is not a simple matter of releasing viral DNA from its protein coat. Studies on the reactivation of inactivated virus suggest that viral protein as well as DNA is necessary to initiate infection. Rabbitpox, heat inactivated at 55°C, has been shown to be reactivated by vaccinia. When heated at 60°C it remains inactive (93). Heat inactivated vaccinia can be reactivated by ectromelia (119). In such systems infective virus of both types is produced and in some cases "recombinants" appear, bearing characteristics of both parent strains (93). Heat at 55°C does not inactivate viral DNA. On the other hand nitrogen mustard can inactivate DNA, and when rabbitpox treated with nitrogen mustard is inoculated onto the chorioallantoic membrane with heat inactivated vaccinia, pock marks characteristic of vaccinia, not rabbitpox are produced (136). Protease inactivated vaccinia can be reactivated by ultra-violet irradiated vaccinia (157). It is worth noting that the effect of inactivated virus on active virus is not always a salubrious one. The multiplicities of each virus modify the outcome. Mixtures of heat inactivated and active vaccinia inoculated into L cells show an inhibition of virus replication if the inactive virus is present in great excess (101). Smaller doses of inactivated virus are less inhibitory. Interestingly, this effect is lost if the inhibiting virus is heated at 60°C instead of 56°C. There seems little doubt that the integrity of viral protein as well as viral DNA determines the initiation and outcome of infection.

Uncoating

Following phagocytosis but before the synthesis of new viral DNA and protein a complex process of uncoating must take place. In normally infected L cells vaccinia virus particles are transferred to the cytoplasm surrounded by the membrane of the phagocytic vesicle (67, 68). This membrane and the outer coat of the virus particle appear to be broken down at the same time. The inner core, which encloses viral DNA, is not susceptible to this initial digestive process. Inactivation by heat or antibody causes the virus particles to remain trapped within the membranes of their phagocytic vesicles where they are ultimately destroyed. Some undenatured or unblocked component(s) of the outer coat seems to be required for the successful transfer of the viral core into the cytoplasm (Fig. 1).

Viral cores gaining access to the cytoplasm reach their peak concentration 1 hour after infection (68). At this time viral cores begin to break down, releasing "naked DNA" into the cytoplasm. Once released, DNA tends to localize in regions of "viroplasm" or "factories" where viral replication takes place. The mechanism of release of "naked DNA" from the viral core remains an unsettled problem. In studies on rabbitpox in Hela cells Joklik suggested that the induction of a new enzyme through the genetic machinery of the host cell was essential for the uncoating of viral cores (137-139). Virus inactivated by either heat or by ultra-violet irradiation could be degraded to viral cores but no further. The inhibition of the uncoating of viral cores can also be

correlated with the inhibition of host cell protein synthesis by puromycin and p-fluorophenylalanine and with the inhibition of DNA-dependent RNA synthesis by actinomycin D. Joklik has concluded that if viral cores are to be broken down, a new enzyme must be synthesized after infection and in response only to undenatured viral protein. This viral protein is released when the phagocytic vesicle and outer viral coat are broken down. This protein causes a derepression of a portion of host cell DNA. Mediated by DNA-dependent RNA, a new enzyme is formed which is instrumental in degrading viral cores, releasing naked DNA (137-139). However, in L cells it has been shown that actinomycin D does not completely inhibit the virus controlled induction of protein (156). Hela cells may be more sensitive to actinomycin D than L cells but the need for cellular DNA in the induction of uncoating enzymes is in doubt. On the basis of their electron-microscopic studies, Dales and Kajioka have concluded that the uncoating of viral DNA is effected by pre-existing enzymes mobilized on the entrance of foreign viral protein (68, 143). Heat inactivated vaccinia does not reach the core stage but is instead degraded inside the phagocytic vesicle. Ultra-violet irradiated virus reaches the core stage normally but is uncoated more slowly. With actinomycin D, virus particles reach the stage of uncoating and form normal cytoplasmic factories, but no infective virus is formed. In this last instance it is apparent that "naked DNA" can be released normally, while the replication cycle is blocked by actinomycin D only at a later stage.

Initiation and the cycle of replication

Having traced the progress of the vaccinia particle from adsorption and phagocytosis to the final release of "naked DNA," we must turn to a consideration of the factors involved in the synthesis viral DNA and protein and the assembly of new infectious virus. Cairns has postulated that one infective particle, the "initiator", accomplishes a critical step in the process of infection (43). In normal suspensions of virus, the production of pocks on CAM shows that there may be anywhere from 12.3 to 97 particles, as counted by the electron microscope, for each pock that is formed (77). Cairns has suggested that the "initiator" activates all of the infecting particles and that each of these in turn sets up a separate center of virus synthesis in the cytoplasm (43). This may not be true in the strictest sense; the "naked DNA" from a number of cores seems to come together in discrete portions of the cytoplasm, the so-called "factories" (68). There is no morphological evidence to show that each factory is set up by only one "initiator"-activated particle. What is clear, however, is the high degree of synchrony that characterizes the synthesis of new virus. All the centers of virus synthesis in the cell start to open up at the same time, and they increase in size, but not in number with time (43). This synchrony is also apparent on the plaque level (164).

A number of studies of the time cycle of vaccinia virus multiplication have been undertaken, utilizing a variety of techniques. With fluorescent antibody and radioautographic techniques in KB cells Cairns

has shown that a period of 3.5 hours elapses before virus protein and DNA synthesis begin (43). Both appear at the same time. The centers of synthesis grow in size and by 9 hours some are fused. Yet it is not until 10 hours that viral DNA and protein are assembled; up until this time viral DNA is susceptible to DNAase. Loh and Riggs using cytochemical and differential fluorescent antibody techniques in Hela cells found an early increase in cytoplasmic RNA, followed shortly by a rise in cytoplasmic DNA at 4 hours (168). Protein antigen appeared at 4 hours, nucleoprotein antigen at 6 hours and by 10 hours they could be found together in the same particle. At this time infective virus was present. Shatkin using metabolic inhibitors in Hela cells found viral DNA synthesis from 1.5 to 7 hours, viral protein from 1.5 to 14 hours, a viral DNA-protein complex from 2.5 to 14 hours and infective virus formation from 5 to 14 hours (231). Other studies have placed the formation of viral DNA within a period of from 0.5 to 6 hours after infection (78, 81, 155, 224, 233). Considering the wide range of techniques and the variety of virus and cell strains the results are reasonably comparable. But there are points of disagreement. Synthesis of viral protein and DNA may begin simultaneously or at separate times; viral DNA-protein complexes may form early or late in the replication cycle.

Cellular DNA and protein

The observation that tritiated thymidine (TdR-H^3) labeled cellular DNA is not transferred to viral DNA demonstrated the need for de novo cytoplasmic synthesis of viral DNA (233). Host cell DNA is preserved

during infection. Cairns found it still functioning at 9 hours (43). Kit and Dubbs found that infection depressed host cell DNA synthesis, but that cellular DNA was not appreciably degraded until after 15.5 hours when the cell was dying (153-155). Ackermann and Loh found a slight increase in cellular DNA during infection (1). The fate of cellular protein has been less well studied; actinomycin D-inhibited viral multiplication allows for continued cellular protein synthesis (212). There are considerable difficulties in separating cytoplasmic protein synthesis into its cellular and virus-specific components, but it is clear that cellular integrity is maintained until the very late stages of infection.

RNA and viral protein synthesis

There is good evidence that all viral protein synthesis is not dependent upon the continuing synthesis of viral DNA. Sodium azide or FUdR inhibition of viral DNA synthesis allows continued synthesis of viral protein (79, 225). Under the electron microscope FUdR and mitomycin C inhibition of viral DNA synthesis allows continued structural protein synthesis but the particles formed are immature and largely devoid of nucleic acid (143). Small amounts of infective virus may be formed, but this is most likely due to a conservation of the original viral DNA, with its incorporation into new particles (82, 143). If viral proteins can be synthesized in the absence of DNA, viral DNA can be synthesized in the absence of certain virus-specific proteins. Actinomycin D inhibition of these proteins allows viral DNA synthesis [illegible] germination. [illegible] found [illegible] [illegible] [illegible] [illegible] synthesis [illegible] [illegible] and that synthesis [illegible] [illegible] hours when the cell [illegible] (211-213). [illegible] [illegible] (4). [illegible] cellular [illegible] less well [illegible] viral multiplication [illegible] (212). There are considerable difficulties in separating [illegible] protein synthesis into the [illegible] it is clear that [illegible] stages of [illegible].

[illegible] and viral protein synthesis.

There is good evidence that all viral protein synthesis is dependent upon the continuing synthesis of [illegible] The inhibition of [illegible] RNA synthesis [illegible] (93, 217). [illegible] synthesis of [illegible] (218). [illegible] (91, 219). [illegible] viral proteins may be [illegible] synthesis [illegible] to continue unimpeded (143).

Lest the impression be created that viral protein and DNA synthesis are entirely independent, it must be remembered that DNA is necessary for the synthesis of RNA. Certain messenger RNA's are responsible for the synthesis of specific proteins, and a viral RNA would be expected to result in the synthesis of specific viral proteins.

Cells infected with vaccinia virus produce RNA (139, 167, 168, 226, 241). Studies in Hela cells have shown an increase in cytoplasmic RNA of from 30-100%, first apparent at 4 hours and maximal at 10 hours. Inhibition of the incorporation of adenine into RNA by 5,6 dichloro-B-D-ribofuranosyl-benzimadazole inhibits vaccinia replication (167). The careful studies of Ackermann and Loh on vaccinia infection of Hela cells in both resting and growth phase have demonstrated an increase of cytoplasmic RNA of 10% in resting and 55% in growing cells over the levels in controls (1). Cytoplasmic protein content is increased 96% and 31% over respective control cells. The RNA formed is a small molecular weight RNA located in the "cell sap." The protein increase is found in particulate fractions of the cytoplasm. In separate studies in Hela cells Joklik and Rodrick found that adenine 8-C^{14} was incorporated into RNA in all fractions of control and vaccinia infected cells (134). Although total amounts of RNA were higher in the nuclear and mitochondrial fractions, only the microsomal fraction showed an increase in the *rate* of incorporation in infected cells. They were unable to determine whether this was due to the increased turnover of pre-existing RNA or

the synthesis of a new species of RNA (135). Very recent studies by Salzman, Shatkin and Sebring have shown that a viral messenger-RNA of high molecular weight appears in cells shortly after infection and by 2.5 to 3.5 hours accounts for 80% of the cytoplasmic RNA (226, 227). This m-RNA has a base composition quite similar in some but not all respects to that of viral DNA, is not associated with ribosomes, and is probably not synthesized by the nuclear RNA-polymerase which forms cellular ribosomal-RNA.

Specific viral proteins

Certain specific viral proteins synthesized during the course of infection may have a structural role. Others may have a functional role as enzymes utilized in the synthesis of other viral components. There may be specific viral proteins without any known structure or function. Numerous qualitative studies on the synthesis of viral proteins have been reported. Recently it has been shown that as many as 15 or more virus-specific proteins may be produced during the cycle of viral replication, but it has not been possible to determine which of these are structural, which are functional, and which possess neither of these activities (5).

Of the proteins which have a functional role, a 2 to 5-fold increase in the activity of cytoplasmic DNA-polymerase has been reported in extracts from vaccinia infected KB cells, but it is not clear whether this is different from similar enzymes found in uninfected cells (110, 111, 178). An increase in the activity of thymidylate kinase has also been reported (178).

The only detailed studies that have been reported on the induction of enzymes following poxvirus infection concern the induction of thymidine kinase (TdR-kinase). This enzyme acts in the phosphorylation of thymidine to form its monophosphate and may be important in the synthesis of viral DNA. McAuslan has found that in HeLa cells infected with rabbitpox and cowpox there is an increase in TdR-kinase activity to 6 times that of control levels (172). This activity is induced within 2 hours after the time of infection, and climbs to peak levels of activity at 9 hours. Ultra-violet irradiation of the virus has no effect on the induction of this enzyme, but does damage a factor that limits the levels of the enzyme after induction. In this instance TdR-kinase activity continues to increase after 9 hours, reaching levels 21 to 24 times those in uninfected controls. However, superinfection with non-irradiated virus can repress this process. The control of this induction and repression of TdR-kinase has been worked out in careful studies using actinomycin D, which inhibits DNA-dependent-RNA synthesis, and puromycin, which permits virus to be uncoated, but blocks subsequent protein synthesis (173). It has been found that both RNA synthesis and protein synthesis are essential for both the induction and repression of TdR-kinase. If the synthesis of RNA is allowed to take place, puromycin can prevent its expression by blocking synthesis of the enzyme.

Similar experiments have been conducted in vaccinia infected L cells. Using mitomycin C, an inhibitor which interferes with DNA template and may prevent the uptake of TdR into new DNA, Kit _et al._ have added

support to the idea that viral DNA-dependent-RNA synthesis is needed for TdR-kinase induction. Suppression of vaccinia DNA synthesis or host cell DNA-dependent-RNA synthesis has no effect on this process (156). They have also studied normal and mutant strains of both cells and virus which lack the ability to induce TdR-kinase. It is apparent that this enzyme can be induced by either the host cell or the virus, and once induced, can be utilized for either host cell or viral DNA synthesis (76).

A protein synthesized during the course of vaccinia infection which may have neither a structural nor a functional role is vaccinial hemagglutin (HA). Loh and Riggs were able to demonstrate its appearance by fluorescent antibody at 10 hours, after mature virus had been formed (168). Certain strains of vaccinia exist which do not produce HA (92) and yet they may be infective in animals and capable of inducing normal amounts of neutralizing antibody (56). Vaccinial HA can be inactivated by specific antibody with no effect on virus multiplication (191). Conversely vaccinia multiplication may be inhibited without appreciable effects on the amounts of HA formed (192). Loh and Riggs have said that HA is simply a by-product of vaccinia infection (168). The problem cannot be dismissed this quickly. It appears that on the CAM, vaccinia infection yields 2 hemagglutinins, one sedimentable HA which is virus specific, the other a "floating" HA which is specific for host tissue, both normal and infected (265). For unknown reasons in some strains of vaccinia the loss of HA has been associated with extraordinary changes

in toxicity (55). At the present time the precise nature and function of vaccinial hemagglutinin remains an enigma.

Assembly and release

Once the essential viral proteins and DNA have been synthesized, they must be assembled into complete infective particles and somehow released from the host cell. The mechanisms involved in virus assembly are poorly understood. Studies with fluorescent antibodies have shown the classical LS and NP antigens to come together only after 8-10 hours have elapsed, and this coincides with the appearance of mature virus (168). There are other studies in agreement (43) but certain proteins may be associated with viral DNA much earlier, before infective virus is present (231). On the morphological level, the most recent electron microscope studies show that late in the replication cycle, the outer coat of the virus particle encloses a diffuse mass of material which contains viral DNA (143). Once this process has been accomplished, this internal material differentiates into the various components of the mature viral particle, the viral core, and the two lateral bodies (Fig. 1). Nothing is known of the biochemical mechanisms involved in this process.

From all that is known of the replication cycle of poxviruses, it is clear that all of the particles multiply at the same rate, reaching maturity together. This has been repeatedly confirmed with numerous strains of virus in many cell systems (78, 100, 187, 193, 201). In all of these studies there has been only a tiny fraction of infective virus released into the medium while cells are still viable. Sonic disruption

of cells allows recovery of practically all of the virus present. There may be two methods for the spread of virus from one cell to another. Noyes and Watson observed antigen containing projections from the cytoplasm of infected cells and suggested a direct cell-to-cell spread through cytoplasmic connections (189). On both CAM and in HeLa cell cultures the addition of neutralizing antibody to the cells shortly after infection does not prevent plaque formation (188, 201). This progresses despite the presence of extracellular antibody.

The other manner in which virus may be released from the cell is through the disruption of the cell itself (100). This is best considered as part of the subject of viral toxicity in general and will be dealt with in the next section.

Toxicity of Poxvirus Infections

In animal virus studies there are few aspects of infection as poorly understood as the pathogenesis of viral toxicity. It is well-recognized that viral multiplication and toxicity to the host cell are not necessarily related. Some viruses may cause cytopathic effects (CPE) in one type of cell but not another. In similar fashion a single cell may be susceptible to the cytotoxic effect of one virus but not another, although the second virus is fully able to replicate. Indeed, inactivated or inhibited viruses may cause cell death without replicating. The wide variations in cell susceptibility and viral toxicity seen *in vitro* are

also found in infections of whole animals and man. The mechanisms of toxicity to the cell and the host may be related to the release of newly replicated infective virus.

Poxvirus toxicity *in vitro*

Studies with very high concentrations of active vaccinia virus have produced toxic effects in L cells (229), mouse and human leucocytes (186), human amnion cells (31) and fetal mouse lung cells (36, 37). The initial changes have been agglutination of the cells, followed later by lysis. In leucocytes inactivated virus is also toxic, but thermally inactivated virus fails to cause CPE in L cells and fetal mouse lung cells. In human amnion cells toxic changes are less severe with inactive than with active virus. Neutralizing antibody given at the time of inoculation may prevent CPE (31, 37, 186). Virus inactivated by ultra-violet irradiation may lose its infectivity but retain its ability to produce toxicity (37, 186). In each of these systems toxic effects have been seen independent of virus multiplication. Studies at lower temperatures and with various metabolic inhibitors have suggested that toxicity is not related to the formation of soluble hemagglutinins (37).

The phenomenon of toxicity has been studied more carefully in embryonic rabbit kidney cells infected with rabbitpox (5). Two effects have been seen. If infective virus is being produced, by 8 hours the cells begin to fuse with one another. This does not occur in the absence of viral multiplication. From 2 to 6 hours, however, the cells become rounded and this occurs even when replication is inhibited by

ultra-violet irradiation of the virus, or by treating the culture with sodium azide or isatin-B-thiosemicarbazone. Rounding of the cells is accompanied by the production of at least 15 soluble viral antigens which are also produced during a normal growth cycle. Viral toxicity therefore seems to be related to a partial virus growth cycle, but whether any one or more of these antigens is responsible for toxic changes has not been determined.

From what has been said above, it does not seem unreasonable to speculate that one or more factors are produced during the cycle of virus replication that might trigger a mechanism in the host cell responsible for toxic rounding. The same or perhaps other factors may trigger the same or other mechanisms which lead to cell lysis and the release of infectious virus. If replication is blocked at a point after this crucial step, cellular changes may continue to run their course in the absence of the production of infectious virus. These considerations all suggest that when toxic or cytopathic effects occur they are the result of a specific process set in motion within the cell by the virus as its replication process unfolds.

Attention has recently centered on the question of whether these effects are due to the activation of cellular lysosomes. Allison and Sandelin studied this problem in monkey kidney cells infected with vaccinia (2, 3). They have assayed levels of 5 lysosomal enzymes: B-glucuronidase, acid phosphatase, acid protease, acid RNAase and acid DNAase. Increased levels of activity for all of these enzymes have

been found at 6 hours, a point in time before the appearance of microscopic evidence of cellular damage. This suggests that complete replication is not necessary for their activation. It is not necessary to invoke the induction of virus-specific enzymes to explain cytotoxic effects. Cellular anoxia, increased acidity secondary to the metabolic effects of viral multiplication, or perhaps a viral protein may serve to activate these lysosomal enzymes. This question has also been studied in a more limited manner by Wolff and Bubel (263). They were unable to find an increase in ß-glucuronidase or acid phosphatase in vaccinia infected guinea pig spleen cells, but their studies are not convincing. The absolute levels of lysosomal enzymes may not be important. What matters is where they are located in the cell, and whether or not they are free to act. Further studies on cellular lysosomes are certain to appear, and it is not too much to expect that they will provide explanations for the release of infective virus from cells and the cytotoxicity of poxvirus infections.

Poxvirus toxicity *in vivo*

Before discussing the problem of poxvirus toxicity in the animal host, it may not be out of place to mention briefly some recent observations on the mechanisms of cellular damage caused by other viruses. In mice and in cultures of mouse liver and spleen cells infected with mouse hepatitis virus, almost complete protection *in vivo* and complete prevention of CPE *in vitro* can be achieved by treatment with the antihistamine diphenhydramine (256, 257). This occurs despite the fact that

in both systems virus multiplies to the same extent as it does in controls. In some way, diphenhydramine protects these cells from a normally lethal infection. This drug is known to inhibit phosphoprotein turnover in cell membranes, and this turnover is directly related to an energy-independent decrease in the transmembrane movement of water and potassium ions (141). In unrelated studies on encephalomyocarditis virus infected L cells it has been shown that early in the course of infection the influx and efflux of potassium ions is increased (87). Initially the intracellular potassium concentration is maintained, but later, shortly before the release of infective virus, gross permeability changes take place and potassium is lost from the cell.

These experiments take on importance in the light of recent work by Boulter, Maber and Bowen on the physiologic disturbances in fatal rabbitpox infections (34). The clinical appearance of dying animals plus the knowledge that virus could be isolated in high titers from the adrenal glands at first suggested that death might be secondary to acute adrenal insufficiency. This was ruled out on the basis of incompatible changes in blood glucose and serum sodium levels. Elevated levels of blood urea nitrogen and serum potassium were seen, suggesting the possibility of renal failure. This too was ruled out. It was found that animals dying with rabbitpox developed a precipitous fall in blood pressure 2 to 5 days after the onset of fever. They either died outright or maintained marginal blood pressures for a day or two before dying. Pressor amines were entirely without effect in these animals, and only

one animal out of many improved after a large dose of cortisone. Serum potassium levels drawn shortly before death revealed striking rises, ranging from 8.5 to 31.6 milliequivalents per liter. The mean serum potassium level for normal rabbits is 3.3 meq/liter; in fatally infected animals it was 15.2 meq/liter.

In poxvirus infections there is reasonable evidence to suggest that the degree of viremia reflects the severity of the illness and may indeed indicate the prognosis. Downie and his group were the first to make this correlation (74). In smallpox it was found that if virus were present in the blood at a concentration of 10^4 CAM infecting doses or more per milliliter on the second day of illness, viremia would persist and the patients would probably die. If virus were present at 10^2 infecting doses/ml or less, viremia would disappear and patients would recover. More recently Boulter and Westwood have made similar observations in rabbitpox (262). Animals dying before the eighth day of fulminant infection had higher titers of virus in their blood than those dying later, and in the few animals which survived, viremia was low in titer and irregular.

From these few observations on viremia, serum potassium levels and shock, and from corresponding findings in vitro on cell toxicity resulting from a partial virus growth cycle, the possible activation of lysosomes, and the loss of intracellular potassium, one may speculate, as Westwood has recently done (262), on the events which precede death from poxvirus infections. After the infection of a cell by a poxvirus, a process is set in motion that eventually results in the death of the cell. Ordinarily

this process is irreversible. It may be mediated by cellular lysosomes, and it may occur when the infecting virus is unable to complete it own replication cycle and form new virus. This process of cell death is accompanied by changes in the cell membrane that lead to a loss of intracellular potassium. In some instances potassium loss and cell death may be prevented despite virus multiplication by the presence of membrane stabilizing agents such as antihistamines.

In the animal host and in man, unchecked virus multiplication leads to widespread cellular necrosis, with the release of large amounts of virus and potassium into the circulation. Large amounts of virus in the circulation may infect the capillary endothelium, or more probably littoral cells found in association with capillary endothelium. The loss of capillary integrity following infection may be responsible for the loss of large amounts of fluid into interstitial spaces, giving rise to the vesicles seen in the skin, but more importantly causing severe hemoconcentration and hypovolemia. Shock appears secondary to hypovolemia, and the damaged peripheral vasculature is unable to respond to pressor amines. Of perhaps equal importance in the terminal shock syndrome, large amounts of circulating potassium may have a direct toxic effect on the myocardium, causing either cardiac arrest or fibrillation with death.

There is hardly any direct evidence supporting these speculations. On the other hand there is nothing known which is incompatible with these mechanisms. The problem of virus toxicity in the animal host demands

intensive investigation, but if the literature is any indication, it is being neglected by all but a handful of investigators (34, 262). More must be known of fluid and electrolyte changes such as hypovolemia and hyperkalemia, and of changes in the cells vital organs such as the liver and heart.

Poxvirus infections are not invariably fatal. In both experimental animals and in man the toxic changes discussed in this section may not be as severe or may not occur at all. The result is spontaneous recovery. The host must possess its own mechanisms for resistance and recovery from poxvirus infection.

Resistance and Recovery in Poxvirus Infections

Up until very recent times, the host defense against poxvirus infection has been discussed almost solely in terms of antibody mediated mechanisms. It has only been within the last 5-8 years that other mechanisms have been proposed which either complement or perhaps act independent of the classical antibody systems (19). The stimulus for much of this work has come from clinical observations but an understanding of resistance and recovery from poxvirus infections has come largely from experimental work with vaccinia in a variety of *in vivo* and *in vitro* systems. Discussion will focus on the roles that antibody, delayed hypersensitivity and interferon play in resistance and recovery from poxvirus infection.

Antibody

Standard teaching on the subject of recovery and resistance to vaccinia infection until a few years ago dwelt solely upon the role that antibody played in this process. It is quite clear that resistance to vaccinia infection can be passively transferred from one animal to another with immune serum. It seems logical to assume that persistance of neutralizing antibodies plays a major role in the prevention of reinfection and it has often been observed that virus titers in primary infections begin to fall as antibody makes its appearance (88-90). Yet a definition of the precise identity of this neutralizing or protective antibody has only recently been attempted. Specific antibodies corresponding to the classical vaccinial antigens, the LS or labile-stabile soluble protein antigen, the NP or nucleoprotein antigen and the HA or hemagglutinin, are all distinct from neutralizing antibody. Recent studies have shown that a larger number of soluble antigens are formed in cells infected with vaccinia (5) and at least one type, the HA antigen, may be composed of a host specific as well as a virus specific hemagglutinin (265).

The nature of the specific protective antigen in poxvirus infections has recently been studied by Appleyard and Westwood (6, 7). Neutralizing antibody is produced in highest titer after infection with active rabbitpox and vaccinia virus, yet inactivated virus is also capable of inducing the production of antibody. Soluble antigens with a molecular weight of 100-200,000 have been prepared from both viruses using differential centrifugation and filtration techniques. These antigens are capable of

blocking the activity of neutralizing antibody. Given subcutaneously with adjuvant, or intravenously without adjuvant they are able to stimulate the formation of neutralizing antibody in titers equal to or greater than those following infection when tested *in vitro*. Yet when a direct comparison is made *in vivo* by challenging the immunity induced by either soluble antigen or previous infection, no correlation can be found between the titers of antibody and the immunity present. Actual protection is better following infection. This may indicate that factors other than circulating antibody are concerned with protection against infection.

Evidence that antibody is not solely responsible for protection has come from two sides. The addition of antibody to vaccinia-infected cultures of mononuclear cells does not cause permanent inactivation of the virus (96). It is known that even a large excess of antibody may fail to inactivate a small fraction of virus. Observations in man have also shown that large doses of hyperimmune gamma globulin may fail to alter the course of progressive vaccinia (62) and indeed this condition has been observed in a patient with good titers of his own neutralizing antibody (190). If resistance can be absent in the presence of antibody, it is equally clear that resistance may exist in the absence of antibody. This observation was first made in guinea pigs infected with vaccinia (72) and more recent studies in the same animals treated with doses of radiation sufficient to suppress their antibody response for 28 days have shown that complete recovery can occur within 4-11 days (98). In man it is well known that resistance to a wide variety of viral infections

may occur in hypo- or agammaglobulinemic patients (107). The existence of true agammaglobulinemia has recently been called into question and it seems that these patients may be capable of producing normal antibody, although in greatly reduced amounts (17). This important observation, however, should not be allowed to overshadow the well-documented dissociation of antibody levels and the degree of resistance to infection.

There can be no doubt that neutralizing antibody has an important function in poxvirus infection. It does not appear to affect the uptake of virus by cells (221) yet it may prevent the phagocytized virus particle from escaping its surrounding vacuole (68). If antibody fails to prevent infection, it may be due in part to direct cell-to-cell spread by the virus, thus avoiding exposure to circulating antibody (188). Yet this does not account for the resistance seen in the absence of antibody, and it is quite evident that other mechanisms are involved.

Delayed hypersensitivity

Resistance to infection may be mediated by cellular as well as humoral factors. Dienes and Naterman were the first to observe that guinea pigs infected with vaccinia began to heal their lesions after 4 days (72). By 6 or 7 days their skins showed the ability to respond to intradermal vaccinia with a typical tuberculin-type reaction characterized by a dense mononuclear cell infiltrate. Antibody did not appear until 12 days. In recent years Uhr, Salvin and Pappenheimer have shown quite clearly that it is possible to induce delayed hypersensitivity to purified antigens well before the appearance of corresponding antibodies (255).

may occur in spite of [illegible] (??). The [illegible] of true agammaglobulinemia [illegible] seems that these patients may be capable of producing [illegible] although in greatly reduced amounts [illegible]. The [illegible] however, should not be [illegible] action of accessory factors and the degree of [illegible].

There can be no doubt that [illegible] function in recovery [illegible]. It [illegible] of virus by cells [illegible] but it [illegible] cells from [illegible] natural infection, it may be [illegible] by the virus, thus [illegible] this does not account for the [illegible] and it is quite evident that [illegible].

[illegible]

[illegible] normal [illegible] 12 days. In [illegible]

Using similar techniques in guinea pigs, Turk and his co-workers have demonstrated that the lesion of revaccination is one of delayed hypersensitivity and appears in the absence of antibody (251). This reaction effects a slight but significant fall in the titer of virus isolated from these lesions as compared to the primary lesions. Of considerable interest has been the nonspecificity of this reaction. Animals sensitized to PPD and challenged with vaccinia have shown decreased titers of virus.

Pincus and Flick have extended these observations to the primary lesions themselves. Children revaccinated with live vaccine two days after primary vaccination develop accelerated responses that are identical with the primary response in all respects except size (198). Hypersensitivity to an inactivated virus preparation can be seen as early as 4 days after the initial infection. This suggests that even the primary response to vaccinia is one of hypersensitivity. Newborn rabbits made tolerant to vaccinia with an inactivated preparation later fail to develop a local reaction to both viral antigen and to live vaccinia, and infection is associated with a high mortality (95). In guinea pigs specific antimononuclear cell serum but not pre-immune serum can prevent the development of a primary reaction to active vaccinia and a delayed reaction to inactive virus and also bovine serum globulin and dinitrochlorobenzene (197). The nonspecificity of this response has also been suggested in experiments where inbred strains of rabbits with varying degrees of tuberculin hypersensitivity were tested with vaccinia (199). Vaccination and testing with inactivated virus were followed in one week

by first BCG and then challenge with old tuberculin. In all cases the timing and severity of the two types of lesions were similar. Animals with an inbred resistance to tuberculosis behaved as if they were inbred for vaccinia resistance. Animals more susceptible to tuberculosis lost weight following vaccination and some died.

The cells responsible for delayed hypersensitivity reactions seem to include macrophages. Their singular importance in determining the outcome of poxvirus infections has been discussed in a previous section. Of interest then are observations which suggest that the findings mentioned above are not simply manifestations of local cutaneous reactivity. Rabbit spleen and kidney cells taken from immune and non-immune animals, cultured *in vitro* and then challenged 2 to 3 days later with vaccinia show similar yields of infective virus from kidney cells but a significantly lower yield from immunized spleen cells (243). Antibody has no role in this system and the immunity is specific; challenge with herpes simplex produces no differences in yields. The resistance of the immunized spleen cell cultures disappears after 7 to 8 days when macrophages are largely overgrown by fibroblasts (216). Other studies have shown that mouse macrophages are more susceptible to infection by a virulent than an attenuated strain of ectromelia (215, 216). Macrophages from both susceptible and resistant mice are equally susceptible to virulent strains of ectromelia *in vitro*. However, immune macrophages more readily phagocytize virus, and this property is independent of antibody.

How the resistance of immune macrophages and the strain differences

in response to infection demonstrated by Pincus and Flick are related to the notion that genetic differences in susceptibility to infection operate on the macrophage level is a problem which should continue to challenge investigators (108).

Interferon

The discovery of interferon in 1957 by Isaacs and Lindemann has opened a new era in the understanding of resistance and recovery from viral infections (126). At the present time knowledge in this area is in a state of flux, but certain basic principles concerning the nature and activity of interferon are well established and have been discussed in a number of recent reviews (124, 129, 258). Interferon is a protein with specific antiviral activity, produced by a wide variety of different types of mammalian and even avian cells tested both *in vitro* and *in vivo*. It has a molecular weight of roughly 20-34,000 and as a protein is non-dialyzable and inactivated by trypsin. It is relatively stable to heat and remains fully active after exposure to both high and low pH. The physiochemical properties of interferon vary somewhat, depending upon the cell producing it. Its biological activity is subject to less variation. It is induced by both active as well as inactivated virus. With active virus, interferon may be formed even though the virus does not replicate (105). Recent studies have shown that nucleic acids from animal tissue, rickettsiae, bacteria yeasts and plants and even the polysaccharide statolin are capable of inducing the production of interferon (124). Isaacs has suggested that induction of interferon may be in response to infection [illegible] leading to the notion that [illegible] of susceptibility to [illegible] disease on the individual level is a problem which awaits answers or further investigation (118).

Interferon

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The mechanism whereby interferon production is initiated in cells is not fully understood. In vitro studies of cells in tissue culture infected with eastern equine encephalitis virus in the presence of actinomycin D have shown that interferon production is suppressed, while the virus freely multiplies (124). Actinomycin D inhibits the production of DNA-dependent RNA and Ho has suggested that interferon production in this system is independent of viral protein synthesis and dependent upon an intact cell genome. The induction of interferon must occur early in the replication cycle, for actinomycin D added 3 hours after infection does not block interferon production. When protein synthesis is inhibited by puromycin or p-fluorophenylalanine interferon production is also suppressed but it is not know whether these agents block the synthesis of interferon itself, or other proteins necessary for its synthesis (124, 129).

The interferon formed in infected cells is released by an unknown mechanism. However, its antiviral activity cannot be demonstrated extracellularly and it does not appear to affect the adsorption or release

of virus from cells (129). There is a possibility that interferon may in some way be related to cell receptor sites (124). Interferon seems to act after the uncoating of the virus within the cell. It was initially suggested by Isaacs, Porterfield and Baron that interferon might uncouple oxidative-phosphorylation within the cell, depriving virus particles of the energy necessary for their replication (15, 128). There does seem to be good correspondence between the inability of viruses to grow at lower oxygen or higher carbon dioxide tensions and their sensitivity to interferon (129). But more recent studies with dinitrophenol, a known uncoupler of oxidative-phosphorylation, have not supported this hypothesis and have shown instead that interferon acts on a non-DNA-dependent-RNA. This may be either new or normally present in the cell and either specifically involved in virus synthesis or more sensitive to interferon (162).

There also seems to be good correspondence between virus virulence, its optimal temperature for growth and both its ability to induce and its sensitivity to interferon. The studies with poxviruses are of special interest here. Bedson and Dumbell have shown that the higher the ceiling temperature at which pocks are produced on the CAM, the more virulent the virus is for the particular host (29). For example, variola major has a higher ceiling temperature than alastrium, the highly virulent Utrecht strain of rabbitpox produces pocks at 2°C above the normal temperature of its host, and the more virulent strains of vaccinia usually have higher ceiling temperatures than less virulent

strains. Later studies by other workers with a number of viruses including vaccinia have shown that virulence is associated with higher temperatures for optimal growth (218). The more virulent viruses not only produce less interferon, they are also less sensitive to the activity of exogenous interferon (219). Interferon production in any one system has also been shown to be greater at temperatures above the optimal growth temperatures for each particular virus.

It is not known whether a virulent virus is able to avoid stimulating interferon production or if it actively blocks its production or action. But it does appear that with certain viruses, among them Newcastle Disease virus, the addition of active virus to cultures which have been stimulated to produce interferon by the same, inactivated virus, promptly shuts off the production of interferon (124, 129). This phenomenon of "inverse interference" remains obscure, but it seems generally true that viruses either stimulate interferon without multiplying, or inhibit interferon production and multiply. This delicate balance has been demonstrated in two excellent studies. A small number of good interferon producing cells can protect a culture made up largely of more virus susceptible cells that are poor interferon producers, unless the challenge dose of virus is too strong (114). And in a superbly conceived experiment, Glasgow and Habel were able to maintain cultures of mouse embryo cells infected with vaccinia for 3 to 5 months by varying the concentration of interferon in the medium (104). If interferon were allowed to accumulate, the cultures spontaneously recovered; if it was

removed too frequently, the cultures were destroyed. These latter findings would appear to be in agreement with observations in vaccinia infected chick embryo fibroblasts where carefully controlled amounts of interferon delayed the growth rate of plaques but did not reduce their eventual number or size (103). Whether interferon reduced the total number of particles formed or extended the time necessary for viral replication is not known.

With such clear-cut antiviral activity in a variety of *in vitro* systems, it is not at all surprising that interferon has demonstrated similar capacities against vaccinia infections *in vivo*. That it may be solely responsible for resistance and recovery from primary vaccinial infections was first suggested by Baron and Isaacs (14). It had been known that chick embryos were highly susceptible to vaccinial infections until they were 7 to 10 days old, when they began to develop resistance. Chick embryos lack both the ability to form antibody and to develop delayed hypersensitivity. Their resistance to infection was directly related to their increased ability to produce interferon. Friedman and his co-workers have extended these observations to guinea pigs (99). Animals whose ability to form antibody and develop delayed hypersensitivity had been totally supressed with Methotrexate and x-ray, recovered from intradermal vaccinia infections in the same manner as did the unsuppressed but similarly infected controls. Virus disappeared from all tissues at the same rate, and interferon assays of the lesions were roughly the same for all groups.

The *in vivo* effects of interferon were first demonstrated with vaccinia in rabbits (163). Interferon prepared in rabbit kidneys has

been shown to protect against intradermal vaccinia when injected locally (127). Protection against larger doses of viruses can be achieved only by injecting interferon 24 hours before infection. Interferon prepared in chick cells and monkey kidney cells also provides a high degree of protection, the necrosis usually following infection being greatly inhibited or completely prevented (4). Rabbit kidney interferon can also delay or supress the lesions of vaccinial keratitis in rabbits (54, 140).

It might be mentioned at this point that intradermal vaccinia in rabbits has provided at least part of the answer to the question of how cortisone inhibits recovery from viral infection. When applied topically to lesions, or given systemically before infection, cortisone significantly impairs resistance to vaccinia (39, 53). Treated animals show less local reaction but the lesions persist longer than in controls. Virus is usually found in higher titer locally and systemic involvement is more severe (39). It is now known that cortisone, which can inhibit the formation of interferon in other systems, inhibits the normal protective effect of preformed interferon on vaccinial lesions (152). It is probable that the general effects of cortisone upon the local inflammatory response procede independent of its effect on interferon.

In man, interferon also seems to play an important role in the body's defense against viruses. Baron and Isaacs have demonstrated the complete absence of interferon in the lungs of patients dying with influenzal pneumonia (16). The absence of control specimens scarcely lessens the importance of their observations. With vaccinia, low titers

of interferon prepared in monkey kidneys and injected locally failed to halt the spread of progressive vaccinia in an infant (62), but more promising results have been obtained with similar interferon in a vaccination trial (230). After receiving interferon in one arm, 38 volunteers were vaccinated in both arms 24 hours later. With each patient serving as his own control, the interferon treated arms developed 14 lesions, the control arms 37, thus indicating a significant degree of protection by interferon.

This discussion will close with a consideration of circulating interferon. In all of the in vitro systems considered thus far, interferon has been released only after a number of hours, often 6 or 7. It is of great interest then that large doses of infectious virus, including vaccinia, when inoculated intravenously into mice produce detectable serum levels of interferon after only one hour and peak titers after only 4 to 4.5 hours (18). Kono and Ho have confirmed and extended these observations (124, 158). Working with Sindbis virus in rabbits, they found serum interferon after 1 hour with peak titers at 7 hours. The same virus in rabbit kidney monolayers produces detectable interferon from 7-24 hours after infection, but not before. Virus replication occurs after 3 hours. To determine the source of this circulating interferon, liver and spleen cell suspensions were incubated with virus and interferon was shown to be released after only 3 hours. Cells of the reticulo-endothelial system seem to be the source of this rapidly released interferon. It is not yet clear whether this represents

totally new synthesis of interferon, rapid completion of synthesis from high concentrations of precursors, or release of preformed complete interferon. It is of interest that thorotrast partially inhibits the release of rapid interferon. Ho has suggested this rapid interferon may prevent early viremia and possibly death (124). Confirmation of these findings must be sought in experimental poxvirus infections, and it will be of great interest to know whether interferon can be detected in the serum of patients with smallpox, and if so, whether it is in low titer or absent in those who go on to death.

III. CHEMOTHERAPY OF POXVIRUS INFECTIONS

Thiosemicarbazones

Antiviral activity in experimental poxvirus infections *in vivo*

It has been fifteen years since the initial observation by Hamre, Bernstein and Donovick that specific chemotherapy of poxvirus infections was a real possibility (117). The availability of the thiosemicarbazone compounds, then being used experimentally against tuberculosis, prompted these workers to use p-amino-benzaldehyde-3-thiosemicarbazone against vaccinia infection on the chick chorioallantoic membrane (CAM). Although complete protection was not achieved, there was a significant delay in death of the infected embryos. Encouraged by these results, mice were next infected intranasally with vaccinia and treated subcutaneously or orally. Again deaths were delayed (117, 118). Protection could be achieved in a few animals when the drug was delayed five days after infection, but best results occurred when animals were pre-treated for two days, then for ten days following infection with vaccinia. In this latter group there were some survivors. Thiosemicarbazones exhibited no anti-vaccinial activity when they were incubated with virus *in vitro* and then removed before infection. The drugs seemed to act within the infected cell itself.

These observations were confirmed and extended by Thompson and his co-workers using first non-substituted benzaldehyde-3-thiosemicarbazone

both on CAM and in intracerebrally infected mice (249). Various substituted thiosemicarbazones, especially isatin-B-thiosemicarbazone (ITSC), also conferred protection (248, Fig. 2a) and the sulfur moeity was shown to be essential for activity, the semicarbazones having no antiviral effect. When fed in the diet at 0.6% concentration, ITSC given as late as 48 hours after infection gave some protection. Dichlorophenoxythiouracil, which alone had only mild antiviral activity, increased the number of survivors when given together with ITSC (185, 250). In these experiments, two important observations were made. First, the protection against vaccinia was primarily a delay in death, although a number of animals managed to survive. Tests for the growth of virus in brains of infected mice showed that treatment had failed to prevent the multiplication of virus. Virus concentrations eventually reached a maximum plateau and death occurred within the next 48 hours. In some mice, titers of virus reached levels that were not tolerated in untreated mice (247). Secondly, thiosemicarbazones appeared to offer no protection in rabbits infected with vaccinia. In six rabbits infected intradermally with the IHD strain of vaccinia, benzaldehyde thiosemicarbazone and cinchoninaldehyde thiosemicarbazone were given intraperitoneally in single daily doses of 100 to 150mg for 6 to 8 days. From their observations, Thompson and his co-workers concluded that therapy did not prevent the formation of lesions at the site of inoculation, "although generalization of infection and development of necrosis at the site of the lesion were prevented" (247). An unreported number of rabbits were also infected intracerebrally.

and here ITSC, given in 3 doses of 250mg/kg over a 48-hour period, failed to give protection (247). These limited and poorly documented studies created the unfortunate impression that thiosemicarbazones have no effect in rabbits. Numerous citations of these studies in the recent literature attest to this fact, and the result has been a total neglect of the vaccinia-infected rabbit as a system in which further studies of the antivrial activities of thiosemicarbazones could be carried out (20, 80, 245, 246). This has been all the more unfortunate since later studies by Bauer have demonstrated a far higher degree of antiviral activity against vaccinia than was seen by Thompson and his group (20).

In the last decade, studies on the thiosemicarbazones have concentrated on ITSC and its substituted derivatives. Bauer was the first to show that ITSC given 125mg/kg subcutaneously twice daily gave complete protection against death in mice infected intracerebrally with up to 10,000 LD_{50} of vaccinia (20). Virus was still able to multiply but at a titer 1 log unit less than in untreated infected controls, and was probably responsible for the subsequent immunity shown in surviving mice to a challenge dose of 100 LD_{50} of the same virus. More recent studies have shown that as little as 2mg of ITSC given subcutaneously twice daily for 4 to 5 days protects infant mice against intracerebral infection with up to 100,000 LD_{50} of the Utrecht strain of rabbitpox (21). These animals develop immunity not only to rabbitpox but also to vaccinia. A most interesting finding has been the inability of ITSC to protect mice against ectromelia (24, 27). However, the modified 4':4' diallylthiosemi-

carbazones of isatin or its N-alkyl-substituted derivatives give excellent protection against 100 LD_{50} of ectromelia but are ineffective against vaccinia. Of great importance has been the demonstration that infant mice, uniformly susceptible to fatal infection when inoculated intracerebrally with 100 LD_{50} of alastrim, are almost completely protected by N-ethyl-ITSC in doses up to 25mg/kg subcutaneously given once daily for 5 days (25). When inoculated with 100 LD_{50} of the Harvey strain of variola major, similar doses of ITSC, N-methyl- and N-ethyl-ITSC give complete protection (25, Fig. 2c). In the mouse-alastrim system, N-ethyl-ITSC has a therapeutic index of 2000 (23).

Structure-activity relationships

The initial studies with thiosemicarbazones by Thompson and his co-workers demonstrated that antiviral activity was dependent upon the thiosemicarbazone (TSC) side chain and a cyclic component, other molecular modifications showing no antiviral activity (248). The curious specificity of different thiosemicarbazones for vaccinia and ectromelia has led to extensive investigations by Bauer and Sadler to determine which particular molecular structures are essential for antiviral activity (22, 222).

The basic structure of isatin-β-thiosemicarbazone is shown in Fig. 2a. Numerous derivatives of ITSC have been assayed against the IHD strain of vaccinia given intracerebrally in mice, and the results indicate that:

1. Modifications at positions 2 and 3 inactivate the compound.
2. Substitutions on the aromatic ring cause a loss or a reduction in antiviral activity greatest at position 5, less at 4 and least

at 7.

3. Substitutions on position 1 have a different effect. If the relative activity of the parent compound ITSC is designated 100, N-methyl substitution has an activity of 202 (Fig. 2c); N-ethyl, 286; and 2-hydroxyethyl, 204. If the side chain is shorter (e.g. acetyl), or longer (e.g. propyl), or if it is more polar there is a decrease in activity.
4. Modification of the pyrrolidine ring has shown that an all planar configuration is necessary, and that the orientation of the $- CSNH_2$ group relative to the ring nitrogen is critical. Substitutions on this side chain result in a loss of activity. These results suggest that antiviral activity is in some way dependent upon the presence of an intramolecular, 6-membered hydrogen bonded ring (Fig 2b). Intramolecular hydrogen bonding is also characteristic of those members of the benzimidazole thiosemicarbazone group possessing antiviral activity.
5. There is some correlation between the lipid solubility of ITSC or its derivatives and their antiviral activity. Compounds that are readily water soluble are inactive. The inability of active compounds to dissolve in aqueous solution has created a significant problem in chemotherapy. The agent must be delivered in suspension when given subcutaneously. ITSC has been found to be active when given orally, so this has not been as unfortunate as might have been expected.

6. ITSC does not cyclize *in vivo* to form an indolothiotriazine, which might act as an anti-metabolite, nor is it hydrolysed to isatin which might act as a dehydrogenase. There is no evidence to support its acting as a purine, tryptophane or serotonin antagonist. Its structure does not suggest an anti-nicotinamide effect and its activity does not seem dependent upon its chelating abilities.
7. The specific requirements for antiviral activity against vaccinia and ectromelia suggest a very narrow range of activity for each compound which is dependent upon certain structural characteristics of each virus.

ITSC in tissue culture and possible mechanism of action

Having knowledge of which structural specificities are necessary for antiviral activity does not answer the question of how this activity is expressed. *In vitro* studies with tissue cultures have helped provide some insight into the mechanism of antiviral activity of ITSC. As would be expected from the studies in mice, vaccinia, rabbitpox and to a lesser degree ectromelia-infected tissue cultures are susceptible to ITSC (233). Embryonic rabbit kidney cells are able to tolerate ITSC in concentrations up to 40uM/ml and at this level are protected against $10^{4.7}$ $TCID_{50}$ of vaccinia or rabbitpox. Virus is not inactivated *in vitro*, nor is its adsorption to the cell or eclipse interferred with (80). ITSC does not act as a non-specific depressant of cell metabolism; other viruses can grow quite well in the presence of ITSC (234).

There has been no evidence as yet to suggest that the synthesis of viral DNA is inhibited by ITSC. KB cells followed by autoradiography show no decrease in the formation of viral DNA (80). In Hela cells, there is no difference between the TdR-H^3 uptake into DNA in infected cells alone and in infected cells treated with ITSC (11). Inhibition is not secondary to the production of a non-functional DNA; viral-DNA which has accumulated in the cytoplasm in the presence of ITSC may be incorporated into infective virus after the agent is removed (10). If ITSC is added after the synthesis of viral DNA is complete, virus synthesis is still inhibited (11).

With respect to the synthesis of viral protein, earlier studies in vaccinia-infected KB cells using fluorescent antibody techniques suggested that ITSC did not affect the synthesis of viral protein (80). The induction and repression of thymidine kinase and DNA-polymerase are not affected by ITSC (179). One protein not affected by ITSC is the one(s) that may be responsible for cytopathic effect (CPE) following infection. A number of studies have confirmed the observation that although viral production is shut off by ITSC, cell destruction secondary to viral infection occurs regardless of treatment with ITSC (5, 11, 234). Recently, experiments with puromycin in vaccinia-infected Hela cells have shown that inhibition of protein synthesis 1 hour but not 3 hours after infection prevents cytopathic effect (179). The implication is that the critical synthesis of a CPE causing protein between 1 and 3 hours is not affected by ITSC.

The antiviral effect of ITSC seems to appear near the end of the replication cycle, and although perhaps all viral components are synthesized, they cannot be assembled into a mature particle (Fig. 7). There does not seem to be any synthesis of intact but non-infectious particles (11). The mechanism by which assembly is blocked is not fully understood, but there are data suggesting certain possibilities. Sadler has recently stated that antiviral activity may be secondary to the intercalation of the planar hydrogen bonded ITSC molecules with viral DNA without impairment of normal cellular function (223). Appleyard and his co-workers have shown that the viral inhibitory activity of ITSC is greatly reduced by the addition of actinomycin D, and completely prevented by the simultaneous presence of fluorophenylalanine, suggesting that both DNA-dependent RNA and protein synthesis are necessary for the action of ITSC (8). If this is so, the intercalation of ITSC with viral DNA must not be such as to prevent the synthesis of DNA-dependent RNA. It is not clear how these findings fit in with the important observation made earlier by Appleyard and his co-workers that in a normal cycle of replication, at least 15 soluble viral antigens are formed. When ITSC is added, it prevents the formation of 4 of the antigens which are among those appearing late in the growth cycle (5). Whether these 4 antigens are necessary for the assembly of mature virus particles is not known.

In summary, ITSC possesses antiviral activity *in vitro* as well as *in vivo*. It does not seem to prevent the synthesis of viral DNA nor of most viral proteins. However, final assembly of the viral particle is

blocked by a mechanism that remains poorly understood.

Clinical use of thiosemicarbazones in man

The successes achieved by the thiosemicarbazones in the suppression of experimental vaccinia infections have quite naturally led to their use in similar infections in man. There have been no reports on the effect of thiosemicarbazones on vaccination "takes." Such observations would not be difficult to make and are needed. Considering the ability of IUdR to suppress vaccination "takes," it would be important to know the activity thiosemicarbazones under the same conditions (49).

It is difficult to evaluate the effects of thiosemicarbazones on more severe vaccinia infections in man. Such cases occur sporadically and it is hard to imagine a large series of patients being observed in any one medical center. The results of therapy in isolated cases reported in the literature make comparison impossible. Eczema vaccinatum may be considered an unfortunate and sometimes serious complication in an otherwise normal patient. N-methyl-isatin-B-thiosemicarbazone (Fig. 2c) has been given in doses of 250mg/kg orally four times daily with apparent success (234). Therapy was later followed by a severe episode of gastroenteritis, but it is not clear whether this was due to drug toxicity. Blood levels of 190ug/100ml were achieved; these levels are similar to those found effective in tissue culture, but measurements may have included inactive or degraded forms of thiosemicarbazone. Progressive vaccinia is a more severe condition, occurring usually in patients with immunological or other defects in body defense mechanisms. N-methyl-

isatin-B-thiosemicarbazone has not been effective in preventing death due to this condition in infants with severe gamma-globulin deficiencies (62, 94). In one patient with leukemia, the lesions of progressive vaccinia remitted following therapy but concomitant administration of gamma globulin makes interpretation of this response difficult (69).

The observation by Bauer that ITSC protected mice against vaccinia while allowing virus multiplication to continue led to the cautious suggestion that ITSC might be effective in the prophylaxis of smallpox, but not in clinical disease (20). Following the demonstration that ITSC protected mice against variola major (25) a study was undertaken to determine the prophylactic effect of N-methyl-isatin-B-thiosemicarbazone in preventing smallpox in contacts of smallpox patients (26). In 1101 treated contacts there were 3 cases of smallpox; in 1126 contacts not treated, there were 78 cases with 12 fatalities. Effective prophylaxis was seen with oral doses as low as 3g twice daily after meals for 4 days. Side effects were limited to nausea and vomiting. The protection conferred by treatment was independent of the immunity acquired as a result of vaccination, whether given in the past or after exposure to the known index case of smallpox.

Although there are no published studies the prophylactic effect of ITSC has not been matched by similar success in the treatment of clinical smallpox. This problem demands further study, and will undoubtedly receive it. However, the apparent lack of success in treating clinical smallpox in no way detracts from the fundamental significance of Bauer's

work. Given early enough, specific chemoprophylaxis against a severe systemic viral disease is possible, and this alone should encourage all who are engaged in the search for antiviral agents.

5-Iodo-2'-deoxyuridine (IUdR)

The synthesis of IUdR was achieved by Prusoff in 1959 (203). The similarity between IUdR and thymidine suggested that it might be used to antagonize the incorporation of thymidine into DNA of mammalian cells (Fig. 3). Progress in the study of this compound as an anti-neoplastic and later antiviral agent and its metabolism and toxicity in experimental systems and in man has been the subject of many reviews (44, 50, 205, 259-261). No attempt will be made to summarize what can readily be found in these articles; only information relevant to the more recent use of IUdR as an antiviral agent will be discussed, and the reader is referred to these papers for more detailed information.

Antiviral activity of IUdR

The ability of IUdR to act as a metabolic antagonist to thymidine in the biosynthesis of DNA led Herrmann to the discovery in 1961 that IUdR and also BUdR inhibited the formation of plaques by DNA-containing vaccinia and herpes simplex viruses, but not by RNA-containing viruses (123). Thymidine reversed this inhibition. These findings were confirmed *in vitro* (151) and therapeutic activity was demonstrated against both vaccinia and herpes simplex when IUdR was added to tissue cultures shortly

after cytopathic effects were visible (165). In the meantime, numerous studies had begun on the activity of IUdR against both of these viruses *in vivo*.

Perhaps because of the serious problem presented by herpetic keratitis in the United States, studies on the antiviral activity of IUdR have concentrated largely on this condition as it is produced experimentally in rabbits and appears clinically in man. Kaufman was the first to report success with IUdR in the treatment of herpetic keratitis in rabbits (146, 147). The drug was given topically every 2 hours for 48 hours. These findings have been independently confirmed in double-blind studies where 0.1% IUdR was given topically every 2 hours for 5 days beginning 48 hours after infection (64), and in other experiments with IUdR, 5-iododeoxycytidine (ICdR), a reservoir for IUdR, and their diacetyl derivatives (196). The careful investigations of Jawetz and his co-workers have shown that in infected control eyes, virus multiplication reaches a maximum at 72 hours, declines beginning on day 6 and is no longer present by day 11 or 12 (133). Steroids prolong the illness (133, 149). Infected eyes treated with IUdR for 7 days yield little virus, but virus titers may rise to even higher titers than in controls once therapy has been discontinued. Other authors have suggested that IUdR has little beneficial effect in rabbits with herpetic keratitis (84).

The use of IUdR in herpetic keratitis in man has met with considerable success. Chronic corneal infection may result in ulceration and blindness; spontaneous cures occur in only 10% of cases, and debridement

or cauterization have been the standard methods of treatment. With topical IUdR Kaufman *et al.* have obtained a high percentage of cures in superficial dendritic ulcers and in stromal ulcers within an average of 3 and 6 days respectively (148). Treatment is usually given every hour or two throughout the day and night for at least 3 to 4 days, often longer. Similar success has been reported in independent and less well controlled studies (63, 122). Less striking results have also been reported by other workers (70, 109, 170).

Little attention has been paid vaccinial keratitis in rabbits. Kaufman and his co-workers treated a small number of superficial and deep stromal ulcerations with 0.1% IUdR topically every 2 hours (150). Therapy began 2 days after infection and was continued for 2 and 4 days respectively. All superficial lesions were clinically well after 48 hours of therapy but virus could be isolated from half of the treated eyes. Stromal lesions were also cured, although again virus was found in over half of the treated eyes. Independent experiments with the same system have reported no therapeutic effect, but therapy was not continued throughout the night (161).

It is not known why deep stromal lesions are more difficult to cure than epithelial lesions. However, it has been shown that after freeze injury, IUdR has no effect upon the rate of epithelial healing but does retard the recovery process in the cornea (200). There is no danger of systemic toxicity when IUdR is applied topically to the eyes (12).

The cutaneous lesions of both herpes simplex and vaccinia are

susceptible to IUdR. With herpes infection in rabbits, skin lesions are prevented by 120mg/kg given subcutaneously every 8 hours for five days (97). In man herpes simplex runs a course of from 7 to 21 days. An 0.1% solution or 0.5% ointment of IUdR applied 4 times daily may cure cutaneous lesions in 1 to 3 days (115) and recently good results have been obtained in a double-blind study using a modified spray gun technique (142). Less dramatic results have been obtained, however, and some workers advise against the use of IUdR in most cases of cutaneous herpes simplex (41).

With cutaneous vaccinia in rabbits, Calabresi, McCollum and Welch demonstrated that protection against as much as 10^5 times as much virus could be achieved with IUdR, 120mg/kg every 8 hours or 60mg/kg every 4 hours for 5 days (45). With these doses there was no evidence of drug toxicity nor was there any depression of the immune response. IUdR was superior to other pyrimidine antagonists including 6-azauridine, cytosine arabinoside and ICdR (48). Lesions were suppressed even when therapy was delayed as long as 48 hours after infection (49). In man similar effects ave been seen. Patients with advanced neoplasms, who had not been vaccinated during the previous 20 years, were treated with therapeutic doses of IUdR or ICdR. When vaccinated, "takes" failed to occur, but when revaccinated 3 weeks after therapy was discontinued, "takes" occurred (49). This effect was not seen in all patients however. In an independent double-blind study 25 healthy patients were vaccinated and then treated topically with 0.1% IUdR ointment 4 times daily begun 24 hours after vaccination (32). No significant therapeutic effect was

seen, in contrast to the good results seen with the same regimen in herpes simplex (115). These results may have been due to the more serious lesions produced by vaccinia, but perhaps of equal importance was the fact that all of the patients tested had been vaccinated within the previous year. The absence of significant effects with IUdR may have been due to the general suppression of all lesions by active immune mechanisms.

Possible mechanisms of action of IUdR

In the past few years the problem of how IUdR exerts its antiviral activity has been the subject of intensive investigation. As it happens, most of these studies have been done with herpes simplex rather than vaccinia as the infecting virus. The results of these studies offer a fascinating complex of mechanisms by which IUdR might act, but at the present time one can only speculate as to whether these same mechanisms are at work when vaccinia multiplication is suppressed by IUdR.

Before the demonstration that IUdR possessed antiviral activity, studies in mammalian cells, primarily neoplastic, had already established a number of mechanisms whereby IUdR interfered with the incorporation of thymidine into DNA (71, 204, 207). The structural similarity of IUdR and thymidine is shown in Fig. 3. The only difference is the substitution of an iodine in the 5-position in place of the methyl group on the thymine mooity of thymidine.

As a metabolic antagonist to thymidine (TdR) IUdR may act at the level of thymidine kinase and prevent the formation of thymidilyic acid (TMP) (Fig. 4). However, both TdR and IUdR are phosphorylated by cellular seen, in contrast to the good results reported with the pure cultures of strep- [illegible] ([illegible]). These results may have been due to the fact that [illegible] produced by [illegible] [illegible] all of the [illegible] and the [illegible] which [illegible]. The absence of significant [illegible] has been [illegible] general suppression of [illegible] by [illegible].

Possible mechanisms of control of [illegible]

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Before the [illegible] a number of [illegible] of [illegible] and mechanisms [illegible] [illegible] bacteria [illegible].

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enzymes to their corresponding mono-, di- and tri-phosphate forms prior to polymerization into DNA (Fig. 4 Legend). Competition between TMP and IUMP for thymidylate kinase may inhibit the formation of TDP (Fig. 4, #2); similarly, competition by IUTP and TTP for DNA-polymerase may inhibit DNA synthesis (Fig. 4, #3).

More recent studies have revealed that TTP not only functions as the immediate precursor for DNA-thymidine, it also acts as a regulator of the formation of TMP. Evidence for its many sites of action has come from a number of studies. TTP seems to inhibit at least four enzymes involved in the synthesis of TMP, including thymidine kinase (207). In addition, both TdR and TTP may act as inhibitors of the enzyme aspartate transcarbamylase early in the cycle of pyrimidine biosynthesis (207). Perhaps IUTP, because of its similarity to TTP, may also act to inhibit these same enzymes, and thus decrease the amount of thymidine available for DNA synthesis (Fig. 4, #4, #5).

It would be important to know whether the enzymes mentioned above are more sensitive to the inhibitory activity of IUdR or its phosphorylated derivatives in infected cells than in uninfected cells. No studies have been reported in vaccinia infections, but Prusoff, Bakhle and Sekely have recently demonstrated no difference in the susceptibility of thymidine kinase or thymidylate kinase induced by herpes simplex to the inhibitory effects of IUdR and IUMP (207). No reports have appeared on the effects on DNA-polymerase, but if, as these studies suggest, the antiviral activity if IUdR is not due primarily to specific inhibition

of enzymes involved in the biosynthesis of DNA-thymine, IUdR may exert its antiviral effects as a consequence of its direct incorporation into viral-DNA, the result being the formation of a "fraudulent DNA" (Fig. 4).

Prusoff and his co-workers have demonstrated that IUdR, as iodouracil (IU), may replace 18% of DNA-thymine in vaccinia (206). This has yet to be demonstrated with herpes simplex, but occurs to a limited extent with pseudo-rabies, a related virus (144). The effects of this substitution on the properties of cellular-DNA include an increased lability to stress and important biological consequences which include an increased rate of mutation, increased errors in protein formation, an inhibition of cellular reproduction and an increased sensitivity to radiation (207). With regard to antiviral activity, the possibility that "fraudulent-DNA" may cause errors in protein formation is of great interest. By comparing the pKa values of thymidine and IUdR Prusoff has calculated that at the normal body pH of 7.4 the proportion of the enolic form of IUdR exceeds that of thymidine by a factor of 34 (205). This would increase the probability of base-pairing errors, guanine being substituted for adenine. As many as 300 genetic sites on DNA could be altered by the incorporation of as little as 1% of IUdR into DNA (207). Resultant errors in the replication of DNA might lead to either viable or non-viable mutants (Fig. 4, #6). Likewise, "fraudulent-DNA" might lead to the coding of similarly fraudulent messenger-RNA and the synthesis of aberrant structural proteins or inactive enzymes (Fig. 4, #7). It is known that resistant strains of vaccinia may appear when allowed to grow in tissue culture in

the presence of sub-lethal concentrations of IUdR (76, 166). Mutant strains of vaccinia may appear in cultures of LM cells treated with IUdR or BUdR (76). These mutant strains are resistant to the antiviral effects of these agents and are deficient in their abilities to induce thymidine and uridine kinase. It would be important to know if this were the result of a coding error. In tissue cultures infected with vaccinia and treated with BUdR, a large amount of non-infectious malformed virus appears (81). BUdR may replace 13% of DNA-thymine. With IUdR, treatment of cells infected either with pseudo-rabies virus (144) or with herpes simplex (238) also results in the formation of structurally imperfect viral particles. These observations raise the questions of whether a virus-specific enzyme is necessary for the normal assembly of viral DNA and protein into mature virus particles, and if so, whether a "fraudulent DNA" results in the production of an inactive "maturation enzyme" or no enzyme at all (207).

Clinical use of IUdR

IUdR is a weakly acidic compound, having a pKa of 8.5. It is relatively unstable to heat and must be sterilized by filtration. Its low solubility necessitates its use in solutions containing no more than 5 to 7mg/ml (259). Because IUdR is broken down in the gastrointestinal tract, and therapeutic doses require large volumes, clinical use in man is limited to intravenous administration.

Initial studies in mice showed that IUdR was rapidly broken down to iodouracil, and then dehalogenated to form uracil and free iodide (208).

These studies have been confirmed in man (259). The rapid degradation and excretion of radioactive I^{131} UdR leads to the appearance of 74% of the radioactivity in the urine at 4 hours, and 91% at 24 hours. Eighty three percent of the total radioactivity appears as free iodide. As a consequence of its rapid breakdown adequate therapeutic blood and tissue levels of IUdR persist no more than 4 hours, and probably less.

The major problem in the clinical use of IUdR is toxicity. Experimental studies in mice have shown the acute lethal dose to be 2.5g/kg (259). Intraperitoneal IUdR given for 13 days produces an LD_{50} of 318mg/kg. If 30mg/kg are given for 8 days, or 10mg per 25g animal for 4 weeks no toxicity occurs (116). In dogs 100mg/kg intraperitoneally produces gastrointestinal and hematologic abnormalities within 10 to 20 days (259). Adult rabbits are able to withstand relatively large doses; 120mg/kg every 8 hours for 5 days has not produced any signs of clinical toxicity (49).

Initial studies of the clinical pharmacology of IUdR in man were reported by Calabresi *et al.* in 1961 (44). Sixteen adult patients with advanced metastatic neoplasms were treated with daily infusions of from 17 to 124mg IUdR/kg over a period of 1 to 3 hours. Total doses over the course of treatment ranged from 60 to 720mg/kg for periods of from 2 to 7 days. Detailed information was presented on 6 of the patients ranging in age from 52 to 70 years. The major toxic manifestations of IUdR therapy were leukopenia, stomatitis and alopecia. The occurrence of each and its relation to the total dose of the drug is summarized in this chart.

adapted from Calabresi *et al.* (44).

Total Dose (mg/kg)	Number of Courses	Leukopenia (less than 4000 or 50% of WBC's initially below 4000	Stomatitis	Alopecia
60-200	4	1	None	None
200-400	4	None	1	1
400-600	9	7	5	7
600-720	7	7	7	6+

+one patient died of his disease before alopecia could have occurred

The first sign of toxicity was stomatitis occurring usually on the 4th to 7th day. Leukopenia usually became evident on the 9th to 14th day, with a return to pre-treatment or normal levels by 3 weeks. Alopecia usually began after 3 weeks and was often severe enough to cause total baldness. Total doses below 400mg/kg caused toxicity in 3 of 8 courses of therapy and these were minimal. Between 400-600mg/kg, toxic signs were very frequent, and occurred in every patient treated at levels above 600mg/kg. In no case, however, was the eventual death of the patient attributable to the drug. Other less important signs of toxicity noted were anorexia, occasional mild nausea, vomiting and diarrhea. Moderate thrombocytopenia was seen twice, and mild iodism, as shown by edema of the mucous membranes, acneform dermatitis and ptyalism, appeared in 3 patients. These subsided when therapy was discontinued.

In the only other large series of patients reported IUdR was given

alone or in combination with 5-fluoro-2'-deoxyuridine (FUdR) (194). The following table summarizes the dose and duration of each course of therapy, and the incidence of side effects.

	40 Patients FUdR 30mg/kg for 4-6 days later 15mg/kg every alternate day: total 120-200mg/kg	39 Patients IUdR 80-100mg/kg per day total dose: 240-600mg/kg	39 Patients IUdR + FUdR 10mg/kg 7.5mg/kg 3 - 5 doses
Stomatitis	9	17	11
Alopecia	2	14	2
Iodism	-	3	-
Nausea	7	4	4
Diarrhea	5	4	8
Leukopenia less than 1000	4	4	7

The toxic effects were most severe in the most debilitated patients, especially in those who had received prior therapy with bone marrow suppressing agents or who had poor renal function. Side effects unique to this group of patients were a severe local reaction with blub formation at the site of infusion in a patient with urticaria pigmentosa, and phlebitis at the site of injection in two other patients. A total of 17 patients had profound toxic effects. Six of these died and all had received combination therapy. They previously had shown severe renal impairment, and their courses were characterized by severe diarrhea, stomatitis, and bone marrow depression with terminal hemorrhage and infection.

Attempts to prevent the major side effects have been made by Calabresi (46). Stomatitis and ipsilateral alopecia were prevented during a course of IUdR therapy by concomitant infusion of 4mg/kg of thymidine into one of the external carotid arteries. Local bone marrow suppression was circumvented by the infusion of 8mg/kg of thymidine into one of the hypogastric arteries (180). Although these interesting studies support present understanding of the locus of action and mechanism of toxicity of IUdR, the methods used would not be practical adjuncts in the treatment of severe viral infections such as smallpox.

While IUdR has decided toxic effects, the limits of safe clinical use seem apparent. Whether large doses would be needed to treat severe systemic viral infections is not known. Preliminary observations in man have shown that vaccination "takes" can be prevented with lower doses (one total dose of 150mg/kg IUdR, another course of 80mg/kg of 5-iodo-2'-deoxycytidine daily for five days) (49). Experimentally it has been shown that the lesions of cutaneous vaccinia in rabbits can be markedly suppressed with doses of 120mg/kg every 8 hours for 5 days without causing toxic side effects (49). It must be admitted that in both instances the disease produced is primarily a local one and does not seem to produce significant systemic toxicity. Of added interest is the recent finding that IUdR can prevent the multiplication of herpes simplex in Hela cells at a concentration 1/10 of that required to inhibit cell reproduction (65). Whether significant antiviral activity occurs with lower sub-toxic doses of IUdR in systemic viral infections such as

smallpox remains to be determined.

The possibility of late toxic complications of IUdR therapy has been discussed in recent reviews (50, 260, 261). The incorporation of IUdR into DNA of normal cells raises questions of subsequent induction of neoplasms, potential genetic damage or infertility. Other complications which might arise during treatment of acute viral illnesses might be the development of IUdR-resistant strains (42, 166). More recently it has been shown that IUdR may inhibit the production of interferon (125). Only further experimental and clinical trials can determine whether these potential complication do occur, and if so, whether they are serious enough to preclude the use of IUdR in the chemotherapy of severe viral infections.

6-Azauridine (AzUR)

Mechanisms of action of AzUR

The present interest in the investigation of AzUR as an antiviral agent is a direct outgrowth of previous attempts to develop specific antagonists to pyrimidine biosynthesis which might be of therapeutic value in neoplastic diseases. Progress in this field has been periodically reviewed, most recently by Welch (50, 66, 121, 205, 261).

The anti-neoplastic activity of AzUR was first shown in experimental mouse tumors (131). It is a remarkably stable compound and despite its close resemblance to uridine it is not susceptible *in vivo* to cleavage

by nucleosidases. The studies of Handschumacher and his group have demonstrated the mechanisms of AzUR activity in mammalian cells (120, 195). The structural similarity between uridine and AzUR are shown in Fig. 5. At physiological pH, the triazine portion of the azauridine molecule is approximately 50% non-ionized, and consequently in steric and ionic configuration it is similar to uridine. Thus it is able to be phosphorylated by uridine kinase to form azauridine-mono-phosphate (AzUMP) (Fig. 6, #1). Once AzUMP has formed, the triazine is predominantly negatively charged and AzUMP now resembles orotidylic acid (OMP, Fig. 5). Thus it is able to inhibit competitively the activity of orotidylic acid decarboxylase (Fig. 6, #2). This enzyme converts orotidylic (OMP) to uridylic acid (UMP) in the pathway of de novo pyrimidine biosynthesis. Inhibition of UMP synthesis by AzUMP is reflected in a decrease in the incorporation of orotic acid and an increase in incorporation of uridine into DNA (195). The utilization of exogenous uridine in pyrimidine biosynthesis is dependent upon uridine kinase (Fig. 6, #1), which acts to form UMP directly, orotidylic acid decarboxylase being by-passed. AzUR alone seems not to affect uridine kinase activity to any significant degree.

More recently, studies have appeared which suggest that AzUR may have additional sites of action. Ordinarily, AzUR inhibition of orotidylic acid decarboxylase causes the accumulation of orotidylic acid, which is cleaved to orotidine and excreted in the urine. Studies following the incorporation of labeled orotic acid into orotidine in AzUR-treated patients have suggested that the large amounts of urinary orotidine

formed can be accounted for only by assuming that AzUR causes an overall increase in pyrimidine biosynthesis (33, 261). Ennis and Lubin have made the important observation in cultures of Sarcoma 180 cells that pyrimidine starvation induced by AzUR results in a 3 to 4-fold increase in the activity of the enzyme aspartate transcarbamylase (83, Fig. 3). This effect is reversed by adding uridine and AzUR simultaneously, suggesting that the induction of aspartate transcarbamylase is under the control of an end product of pyrimidine biosynthesis. It may be that a deficiency of UMP following AzUR inhibition is responsible for the increase in aspartate transcarbamylase activity, but it should be recalled from the previous discussion of IUdR that thymidine (TdR) and thymidine tri-phosphate (TTP) may act as inhibitors of aspartate transcarbamylase (207). Presumably an IUdR-induced deficiency of TTP might cause an increase in the activity of this enzyme. An AzUR-induced deficiency of TTP might also have similar effects. In earlier studies Handschumacher increased the levels of OMP by adding excess orotic acid. Despite the increase in OMP, AzUR inhibition of orotidylic acid decarboxylase was not overcome (120). This might be explained by assuming that OMP strongly inhibits its own synthesis (120). A mechanism such as this might be at varience with the suggestion that AzUR causes an overall increase in pyrimidine biosynthesis.

Studies in microbial systems have suggested that azauridine diphosphate may inhibit polynucleotide phosphorylase but it is not known what significance if any this has in mammalian systems (205).

One of the most encouraging results of these investigations has been the discovery that AzUR exerts an anti-metabolic effect against a small number of experimental and human neoplastic conditions, especially adult leukemia, while having little or no toxic effect on normal cells (47, 50, 86, 121, 261). It is believed that when the *de novo* pathway for pyrimidine biosynthesis is blocked by AzUR, normal cells are capable of obtaining an adequate supply of uridylic acid (UMP) through the phosphorylation of uridine by uridine kinase (Fig. 6, #1). Neoplastic cells unable to form UMP from other sources, and therefore peculiarly dependent upon the *de novo* pathway, would be the only cells seriously affected by AzUR.

As with many other anti-metabolites, a state of resistance or refractoriness develops during treatment with AzUR. In some systems this may be due in classical fashion to the selection of cells that are deficient in uridine kinase, the enzyme which must be present if AzUR is to exert its effect (121). In man, however, resistance or better refractoriness to therapy disappears when AzUR is withdrawn. This refractoriness may be attributable to the induction of increased enzyme activity along the *de novo* pathway of pyrimidine biosynthesis in the presence of AzUR (121, 261). These two properties, slight toxicity and reversible refractoriness, might make AzUR a particularly valuable agent if it were shown to possess antiviral as well as anti-neoplastic activity. The advantage of having an agent with selective toxicity against viruses while being well tolerated by normal cells is obvious. That refractoriness to therapy appears gradually as the activity of normal cellular

enzymes is increased might be of special significance in the treatment of severe viral infections. AzUR might be able to exert its antiviral activity during the acute stage of the illness, thereby decreasing the amount of virus presented to the host defenses. If an overwhelming viremia were prevented, and the host's task of ridding itself of virus made easier, later induction of new enzymes along the *de novo* pathway of pyrimidine biosynthesis during the refractory period might be of little consequence.

Antiviral activity of AzUR

The initial observation on the antiviral activity of AzUR was made by Rada and his group in 1960 (210). In tissue culture and on the chorioallantoic membrane infected with vaccinia AzUR was shown to cause a 75% inhibition of cytopathic effect at a concentration of 1.2 x 10^{-2}M (209, 210). The ratio of toxic to inhibitory concentrations was calculated as 14. No effect was seen with influenza A, Newcastle Disease virus or eastern equine encephalitis virus.

AzUR also has a protective effect in mice infected intracerebrally with 400 LD_{50} of the IHD strain of vaccinia (132). Animals given 2.5mg daily for 9 days died one day later than controls. Similar results were seen with intranasal inoculations. Urethane potentiated the activity of AzUR, and it was suggested it did so by facilitating the passage of AzUR into the brain. In cutaneous vaccinia in rabbits Smejkal demonstrated that 300mg/kg of AzUR given intravenously on each of 3 days markedly inhibited the appearance of lesions without producing any signs of syste-

mic toxicity (236). These results have been confirmed in greater detail by Calabresi et al. in studies comparing the anti-vaccinial activity of a number of pyrimidine nucleosides (48). IUdR was the most effective agent tested. AzUR was roughly half as active as IUdR, but this was significant in this system (52).

Very few biochemical studies have appeared describing the effects of AzUR on vaccinia-infected cells. Rosenbergova and Rada have reported 90% inhibition of vaccinia by 4.1 x 10^{-2}M AzUR (217). By measuring P^{32} uptake into nucleic acids they found that infected but untreated cells showed an increase in the amount of DNA to 184% over control values at 24 hours. There was a slight fall in RNA from 24 to 48 hours. When treated with AzUR, infected cells showed a marked decrease in DNA to levels 45% of normal at 6 hours and 25% of normal at 48 hours. In normal uninfected cells AzUR caused a decrease in RNA P^{32} uptake to 75% of normal at 12 hours. In infected cells AzUR caused an even greater fall in RNA at 6 hours.

An intriguing observation has recently been made on the levels of uridine kinase induced in CAM following infection with vaccinia (211). Enzymatic activity was determined by measuring the amount of AzUR converted to AzUMP. A two-fold increase in uridine kinase activity was observed in infected cells. One is tempted to agree with the author's suggestion that in cells infected with vaccinia the induction of uridine kinase fortuitously provides for greater conversion of AzUR to AzUMP, and hence inhibition of orotidylic acid decarboxylase. If the activity of uridine and [illegible] (13). [illegible] by Goldman et al. [illegible] a number of [illegible] [illegible]. [illegible] tested. Acid was [illegible] in this system [illegible].

Very few biochemical studies [illegible] AsHR on vaccinia-infected cells. [illegible] inhibition of vaccinia by [illegible] 10^{-5} [illegible] this large nucleic acids [illegible] showed an increase in the amount of RNA [illegible] 24 hours. There was a slight [illegible] treated with AsHR infected cells showed [illegible] with maximum of 6 hours and [illegible] infected cells AsHR caused a decrease in [illegible] P^{32} [illegible] at 16 hours. In [illegible] at 6 hours.

An interesting [illegible] uracil kinase induced in [illegible] thymidine activity and [illegible] to AsHR. A two-fold increase in [illegible] in infected cells. [illegible] that in cells infected [illegible] mechanisms for [illegible] inhibition of [illegible] kinase plays a significant role in determining the effect of AzUR on vaccinia, it might be suspected that AzUR would be less active in the system of Dubbs and Kit in which IUdR and BUdR resistant strains of both LM cells and vaccinia show decreased ability to induce thymidine and uridine kinase (76). This system might be useful to determine whether AzUR is capable of using both cell-induced and viral-induced uridine kinase.

Loh has recently reported on the effect of AzUR on the synthesis of vaccinia virus, but details are not available (169).

It should be mentioned that in addition to AzUR several derivatives of azauracil (AzU), in particular 3-methyl-2, 4-dithio-6-azauracil, have shown equal or greater anti-vaccinial activity than AzUR on CAM (237). These derivatives have a surprisingly long duration of activity. However, the presence of the AzU moeity might preclude these compounds from serious consideration as potential antiviral agents in man.

Clinical use of AzUR

For a number of years AzUR has been studied in the treatment of experiment tumors and human neoplastic conditions, especially chronic leukemias. These results have been extensively reviewed, and only the findings which would be of significance in the treatment of viral illness will be considered here (50, 66, 121, 205).

The remarkable absence of toxicity due to AzUR in man and a number of experimental animals has been attributed to the ability of normal cells to use pathways other than the *de novo* pathway for pyrimidine biosynthesis.

Specifically, AzUR has no marked toxicity for the normal hematopoietic system. It has been of some benefit in treating leukemias, and with prolonged use its ability to cause mild anemia has suggested a trial in patients with polycythemia (52). Other clinical indications at present are mycosis fungoides and especially psoriasis (51, 252).

AzUR itself does not have any toxicity for the gastrointestinal tract, but it is not possible to administer this compound orally. Intestinal conversion of AzUR to azauracil (AzU) with absorption of AzU leads to profound central nervous system toxicity. Recently the introduction of 2', 3', 5'-triacetyl-6-azauridine has made oral administration possible (66). This compound is not converted to AzU in the gastrointestinal tract, but is converted to AzUR after absorption.

One of the difficulties of administering AzUR intravenously is that of maintaining therapeutic blood levels for significant lengths of time. After 4 hours 75% is excreted unchanged in the urine; after 8 hours 95% (121). Therapeutic levels may last only 1 or 2 hours. With triacetyl-AzUR renal excretion is prolonged and significant blood levels may be maintained during the 8 hours between doses (66). One other problem that has arisen with regard to the kidney has been the appearance of crystalluria in some patients. Large amounts of orotic acid and orotidine are excreted, and in addition AzUR appears to have a uricosuric effect (66, 121). These problems have not been of major significance in the clinical management of patients, and might not be of significance in patients being treated for acute viral rather than chronic neoplastic

conditions.

AzUR may be administered in large doses without significant toxicity. Continuous daily doses of 600mg/kg or 180mg/kg three times daily intravenously have been well tolerated. Triacetyl-AzUR 270mg/kg orally three times daily is without side effects. In rabbits AzUR has been given in doses as high as 400 to 450mg/kg three times daily without causing toxic effects (52).

In summary, the unique absence of significant toxicity and the convenience of oral administration in its triacetyl form would make AzUR a highly attractive agent if it were shown to possess activity against severe viral infections in man. Compared to the thiosemicarbazones and to IUdR, there have been few studies to demonstrate its activity against poxviruses. In the only direct comparative study, AzUR was less effective than IUdR in suppressing the cutaneous lesions of vaccinia in rabbits (48). These results should not discourage further studies of AzUR in vaccinia and other poxvirus infections. AzUR is considerably less toxic than IUdR, and it may not be necessary to demand complete suppression of virus by an antiviral agent. A reduction in viral titer of 1 or 2 logs has been shown to be of significance in human as well as experimental poxvirus infections (74, 262). Whether AzUR is capable of such activity in these severe systemic infections must be determined.

IV. EXPERIMENT IN THE COMPARATIVE CHEMOTHERAPY OF SYSTEMIC VACCINIA IN RABBITS

Preliminary Considerations

The search for effective chemotherapeutic agents for the treatment of viral diseases has produced three compounds which possess significant antiviral activity against vaccinia. Their possible mechanisms of action are summarized in Fig. 7. 6-azauridine (AzUR) inhibits the conversion of orotidylic to uridylic acid in the *de novo* pathway of pyrimidine biosynthesis and thus may inhibit the formation of viral RNA and protein and possibly viral DNA. 5-iodo-2'-deoxyuridine (IUdR) is an analogue of thymidine and may inhibit a number of enzymes in the *de novo* pathway of pyrimidine biosynthesis.

It is incorporated into viral DNA, and the "fraudulent DNA" that is formed may interfere with the correct coding of viral RNA and the synthesis of viral proteins. If viral particles form, "fraudulent DNA" may cause them to be non-functional. Thiosemicarbazones, specifically isatin-B-thiosemicarbazone (ITSC), appear to prevent the maturation or assembly of the mature virus particle, without altering viral DNA synthesis. Synthesis of a small number of viral proteins, however, seems to be inhibited.

The success of each of these agents against experimental vaccinia, as well as related poxviruses and other DNA-containing viruses has suggested

their potential usefulness in the treatment of human smallpox. Antiviral activity against vaccinia has been demonstrated by each in a number of experimental systems, but there are hardly any data comparing these 3 agents in a single system. Present knowledge of their activity is compared in Fig. 8. The data indicate that:

1. Thiosemicarbazones are highly effective against intracerebral vaccinia in mice (20) but nothing is known about the activity of IUdR and AzUR in this system.
2. In rabbits, IUdR is topically effective in vaccinial keratitis (150); AzUR is not effective (52). Thiosemicarbazones have not been tested.
3. IUdR, and to a lesser degree AzUR, protect against intradermal vaccinia in rabbits (49), but thiosemicarbazones have not been impressive (52).
4. Intracerebral vaccinia in rabbits has received little attention, but thiosemicarbazones have no effect (247).
5. In man, IUdR (49), but not AzUR (52) prevents primary vaccination "takes." A recent study has shown that N-methyl-ITSC does not prevent primary vaccination "takes" but moderately inhibits the size of revaccination lesions (266).
6. In a variety of more severe vaccinial infections only the thiosemicarbazones have been tried, but results are difficult to assess (62, 69, 94, 234).
7. In human smallpox N-methyl-ITSC has a decided effect when given

prophylactically but is ineffective if therapy is delayed until clinical disease has appeared (26).

The need for an effective chemotherapeutic agent in clinical smallpox, and the absence of comparative studies on potentially useful drugs have formed the basis for this study. A severe systemic infection was produced in weanling rabbits by inoculating them intraperitoneally with vaccinia. The chemotherapeutic activities of N-methyl-ITSC, IUdR and AzUR were then compared in this system.

Materials and Methods

Animals

New Zealand weanling rabbits were obtained from a local supplier. Each animal weighed approximately 2kg. The sex of each animal was not noted. They were kept in the main animal colony for 4 days in order to observe them for signs of illness. They were treated prophylactically with sulfamethazine (Sulmet, 10cc/gallon added to the drinking water) to suppress intestinal coccidia (175). Treatment was discontinued 1 day before the beginning of the experiment to allow for urinary excretion of the drug. Animals were fed a normal laboratory diet and were offered water *ad libitum*. Baseline weights and hematocrits were recorded. Total leukocyte counts were determined using the Coulter counter. Serum specimens were obtained for pre-infection controls of subsequent viral assays.

Virus

A strain derived from the Levaditi strain of vaccinia virus was used in all experiments. This virus had previously been passed by intratesticular inoculation in rabbits (38) and subsequently on the chorioallantoic membrane of embryonated eggs. Prior to use, it had been stored at -70°C. Virus was thawed at room temperature. In the preliminary experiment 10^{-2} and 10^{-4} dilutions were prepared using medium 199 supplemented with 2% fetal calf serum (FCS). In the final experiment a 10^{-3} dilution was prepared with sterile normal saline. This dilution is known to contain roughly 3200 tissue culture infective doses ($TCID_{50}$) (174). Within 30 to 45 minutes all animals were inoculated with 1ml of the appropriate dilution. Injections were given intraperitoneally through the left lower quadrant.

Therapy

Beginning at the time of infection, all animals in the final experiment were treated every 8 hours for 5 days or until sacrifice. Injections were given subcutaneously. Animals were divided into 4 groups of 6 and therapy was as follows:

1. The first group served as the infected but untreated controls. Each animal was given sterile normal saline, 5ml every 8 hours.
2. The second group was treated with 6-azauridine (AzUR) dissolved in sterile normal saline at a concentration of 200mg/ml. Previous experiments in rabbits had shown that doses up to 400mg/kg every 8 hours could be given for 5 days without signs of clinical

toxicity (52). It was decided to treat this group with 300mg/kg every 8 hours. The volume of each injection was between 1.5 and 2.0ml.

3. In the third group 5-iodo-2'-deoxyuridine (IUdR) was prepared as a solution containing 5mg/ml in sterile 5% dextrose and water. Previous studies had shown that rabbits could receive up to 120 mg/kg every 8 hours for five days without evidence of toxicity (49). The same dose was used in this experiment. Single injections ranged in volume from 25-30ml.
4. The fourth group was treated with N-methyl-isatin-β-thiosemicarbazone (ITSC). The drug was suspended in 5% dextrose and water at a concentration of 10mg/ml. There are few data in the literature to suggest an appropriate therapeutic dose of thiosemicarbazones in rabbits. Rabbits were not protected against cutaneous vaccinia when given benzaldehyde thiosemicarbazone or cinchoninaldehyde thiosemicarbazone 100-150mg/kg intraperitoneally daily for 6 to 8 days (248). Isatin thiosemicarbazone, given as three 250mg/kg doses intraperitoneally within 48 hours of infection failed to protect rabbits against intracerebral vaccinia (247). No report was made of drug toxicity. Bauer was able to demonstrate a high degree of antiviral activity against intracerebral vaccinia in mice by giving ITSC 125mg/kg subcutaneously twice daily for 5 days (20). N-methyl-isatin-β-thiosemicarbazone has roughly twice the antiviral activity of its parent compound

(22); therefore, a total daily dose of 125mg/kg of the N-methyl derivative would be expected to give similar protection. In the absence of specific data it was assumed that if rabbits were able to tolerate thiosemicarbazones at doses of 100-150mg/kg daily for 6 to 8 days, they could tolerate the same dose of ITSC and half this dose (50-75mg/kg) if given as N-methyl-isatin-B-thiosemicarbazone. It was decided to give each rabbit 60mg/kg daily for 5 days in a schedule of 20mg/kg every 8 hours. Single injections were approximately 2ml in volume.

Course of infection and treatment

Daily observations were made on all animals and their clinical condition was noted. Weights were recorded except on days 5, 8 and 10. In a few animals showing diarrhea, microscopic examinations of the stool for coccidia were made, and in one showing heavy excretion of these ova simple replacement of fluid loss with 60ml of normal saline subcutaneously was followed by clinical improvement. Frequent blood specimens were obtained from each animal from the marginal vein of the ear. One to 2ml was collected. After centrifugation the cells were separated and the serum saved for viral assay. Capillary hematocrits and total leukocyte counts were determined.

Autopsy

Animals were chosen for sacrifice if they appeared clinically ill. When none of the animals in a group appeared ill, selection was arbitrary. Preliminary blood samples were taken from the marginal ear vein for

hematocrit and total leukocyte determinations. The abdomen and anterior thorax were shaved with an electric clipper and scrubbed with alcohol preps. Sterile cardiac puncture was performed, as much blood being withdrawn as possible. Serum was saved for virus assay. Animals were killed with an intracardiac injection of 20-40ml of air. The abdomen and peritoneum were opened aseptically. Observations were recorded on the amount and nature of ascites when present, and on the gross appearance of the visceral organs, particularly the liver and spleen. Cultures of the peritoneal cavity were taken for bacteriological studies and samples of ascites removed where possible for viral assay. Specimens of liver and spleen and also lung and brain were removed. All serum and tissue specimens were stored first at -70°C and later at -20°C.

Preparation of organ specimens

Individual organ specimens of approximately 1cc were ground in the cold in either mortar and pestle or Ten Broeck grinders. Suspensions were made in 9ml of medium 199 supplemented with 2% FCS. After centrifugation in the cold at 2000 rpm for 20-30 minutes, supernatant fractions were pipetted into screw cap vials and stored at -20°C.

Viral assays

Cultures of a continuous line of Green monkey kidney cells grown in monolayers were used for all viral assays. Monkey kidney cells are known to support the growth of vaccinia (100). Stock cells were grown in Roux bottles in 40ml of medium 199 supplemented with 15% FCS, penicillin (100 units/ml) and streptomycin (100ug/ml). When a monolayer had formed,

growth medium was decanted, and the cells washed once with 20-30ml of Hank's bananced salt solution (BSS). An 0.2% solution of trypsin in phosphate buffered saline was added (20-25ml) and after incubation for 10-15 minutes at room temperature, gently decanted. Bottles were then inverted and incubated another 10-15 minutes. Cells were dispersed by vigorous pipetting using small volumes of medium 199, 15% FCS, then brought to a total volume of 40-50ml. 1ml volumes of this cell suspension were inoculated into constricted tissue culture tubes, rubber stoppered and incubated horizontally at 37°C.

When complete monolayers had formed, usually after 3 or 4 days, growth medium was removed and 0.9ml of fresh medium 199 FCS was added for maintenance. Specimens for viral assays were thawed at room temperature. Serial ten-fold dilutions with medium 199 2% FCS were made where indicated. Tubes were inoculated with 0.1ml of the sample to be assayed. Each specimen was assayed in duplicate. Tubes were incubated horizontally at 37°C and light microscopy readings for cytopathic effect (CPE) were made at 24, 48 and 72 hours. The end point chosen was 48 hours. CPE was graded as follows: + = only a few small cytopathic foci present; ++ = approximately 25% of the monolayer destroyed; +++ = 50% destroyed; ++++ = 75% destroyed; +++++ = totally destroyed.

Results of a Preliminary Experiment

In order to determine the dose of virus necessary to cause severe disease when inoculated intraperitoneally in weanling rabbits, 5 animals were inoculated with 1ml of a 10^{-2} dilution of virus and 4 animals with 1ml of a 10^{-4} dilution. Careful observations of the clinical condition of animals were not made, nor were weights taken, but it was apparent that the 5 rabbits which received the 10^{-2} dilution became seriously ill after 2 to 4 days. They were all sacrificed between days 2 and 5. Multiple serum specimens had been taken from each animal during the course of illness and in all of them virus was cultured from undiluted specimens. Of the organs, only brains and livers were assayed. In 3 brains virus was found at 10^{-2} dilution in 2, and at 10^{-1} dilution in the third. The livers of all 5 animals grew out virus in dilutions as high as 10^{-5} and indications were that in at least two of these livers, virus could have been recovered in even higher titers.

In the group of 4 animals that received 1ml of a 10^{-4} dilution of virus, the animals appeared clinically well when sacrificed on days 3, 4, 8 and 17. All undiluted serum specimens were positive for virus, even in the animal killed on day 17. In view of the results to be reported in the final experiment, the presence of virus in the serum is difficult to explain. The brains of the animals killed on days 4 and 8 yielded virus at 10^{-2} dilution, the brain taken on day 3 had virus present at 10^{-1}. The livers of all 4 animals were positive for virus, but at lower titers

than in the above group infected with the 10^{-2} dilution. In none of the liver specimens was virus found in dilutions higher than 10^{-3}, and this represents 100 times less virus than in the more heavily infected group.

Because of the contrast between the severe illness produced in the group given a 10^{-2} inoculum and the clinical well being despite the presence of virus in the group given a 10^{-4} inoculum, it was decided that a 10^{-3} dilution might be sufficient to produce disease severe enough to kill some but not all of a group of untreated animals. In similarly infected but treated animals, it was hoped that therapy would be successful and would be reflected in a suppression or elimination of virus from serum and organ assays.

Results of the Final Experiment

In order to facilitate discussion, animals will be referred to by an abbreviation for the group they were in and the day they were sacrificed or found dead. For example, C-3 will signify a control animal sacrificed on the third day following infection; IUdR-6, an animal treated with IUdR and sacrificed on the sixth day.

Clinical course

From the results of the preliminary experiment, it was expected that acute illness would make its appearance from 2 to 4 days after infection, and this indeed was the case. Animals C-3, C-4, C-5 and C-6 were all severely ill and C-5 died spontaneously while being prepared

for sacrifice. C-9 and C-11, however, appeared clinically well. Because of the appearance of illness between days 3 to 6, this period will hereafter be referred to as the acute period.

In contrast to the controls, all animals in each of the 3 treated groups appeared clinically well during the acute period, and all were well at sacrifice with 3 exceptions. AzUR-9 was found dead, as were two animals treated with IUdR: IUdR-9a and IUdR-9b.

Gross lesions

The presence of ascites, and the gross appearance of the liver and spleen were graded on a 0-++++ basis. Results are summarized in Fig. 9. Observations were not made on C-3 or ITSC-6 and ascites was not recorded for AzUR-9. A definite pattern emerged from these observations. During the acute period C-4, C-5 and C-6 developed severe ascites. They also had significant lesions in their spleens and to a lesser extent in their livers. These consisted of small white or yellow nodules and cyst-like lesions which studded the surface and appeared within the substance of each organ. Spleens in all cases appeared enlarged. The general impression was one of scattered regions of severe necrosis. These gross lesions were similar to those described by Douglas, Smith and Price (73). Of interest were the mild lesions seen in C-9 and the absence of lesions in C-11. There may be an explanation for this finding in C-11. At autopsy a small, soft nodule was found between the skin and the abdominal muscles. This may have been a reaction to the inoculum, and C-11 may have escaped true intraperitoneal infection.

In the treated groups, gross lesions were considerably less severe or absent. It is difficult to say whether any of the differences between groups are significant, except perhaps for the definite ascites in AzUR-5, and the unexplained presence of a large cystic liver in ITSC-8.

Hematology

In rabbits with severe vaccinia infections Douglas *et al.* reported a 50% fall in the total erythrocyte count by the 7th day after infection with a gradual return to normal (73). Total leukocyte counts fell for 2 or 3 days, then rose to maximum values on the 9th day. In all animals in the present experiment, hematocrits and total leukocyte counts were determined on an average of once every 2 days. No consistent pattern of any kind emerged from either set of determinations. These results will not be reported in detail.

Bacteriology

In all animals cultures of the peritoneal cavity were taken immediately upon opening the peritoneum. Samples were streaked on blood agar plates and inoculated into thioglycolate broth. Mims has reported no growth of bacteria from the peritoneal cavities of mice infected intraperitoneally with ectromelia, though he has cautiously refused to rule out pleuropneumonia-like organisms as being responsible for the lesions seen (184). In this experiment a few samples grew out gram-negative organisms but no consistent pattern emerged in which one organism predominated. Unfortunately, a clerical error resulted in the loss of the final report on these cultures. Despite the impression that bacterial

superinfection was not a complication of clinical illness, it cannot be ruled out in the absence of the culture report.

Viral assays

Serum

Assays for the presence of virus in serum specimens from each of the 4 groups were negative in all but one instance. Samples from each animal were taken on an average of once every 2 days. Only in the sample taken immediately before the sacrifice of C-5 was virus found, and it was not present in a 10^{-1} dilution. These results came as a surprise. In the preliminary study virus had been found in the serum of all animals. The reason for this discrepancy is not known, but it is not unusual to find that serum is not a good source for virus. Viremia in vaccinia seems to be associated primarily with the leukocytes (73, 239), and the same condition seems to exist in smallpox (74).

Brain

The results of virus assays of brains is shown in Fig. 10. Virus was found in samples of brain from all control animals sacrificed during the acute period, but not in C-9 or C-11. In the treated groups AzUR- and ITSC-treated animals were entirely free of virus. In the IUdR-treated group virus was found in IUdR-4 and only at 10^{-1} dilution. Three of the 4 control brains had virus titers that were higher than 10^{-1}.

Liver

In Fig. 11 it can be seen that control animals during the acute period yielded virus in dilutions up to 10^{-5}. C-9 and C-11 were free [illegible] was not a [illegible]

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Thus [illegible] 4 groups were [illegible] and were taken [illegible] taken [illegible] before the [illegible] was not present in a [illegible] solution. [illegible] In the preliminary study [illegible] The reason [illegible]

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of virus. Two of the 4 animals in the AzUR-treated group yielded virus; AzUR-5 at a dilution of 10^{-4}. None of the IUdR-treated animals yielded virus. ITSC-5 was the only animal in its group to yield virus, although in low titer.

Spleen

In Fig. 12 the results clearly show that virus was recovered in highest titers from spleen. In the control group C-9 and C-11 were again free of virus, but the animals sacrificed during the acute period yielded virus, at dilutions from 10^{-5} to 10^{-7}. Of the AzUR-treated animals, 3 out of 4 animals in the acute period yielded virus. The spleen from AzUR-9 was lost in a laboratory accident. AzUR-11 was free of virus. Two of the IUdR-treated animals yielded virus; IUdR-4 at a dilution of 10^{-4} and IUdR-9b at 10^{-3}. It should be mentioned that of all the samples of brain, liver and spleen taken after the acute period, virus was recovered only from this one IUdR-treated animal. ITSC-4 and ITSC-5 yielded virus at 10^{-4} and 10^{-5} dilutions but the other 4 animals were free of virus.

Peritoneal fluid and lung

These samples were saved in the event viral assays of brain, liver and spleen would be inconclusive. On the basis of the results of assays of these organs, it was decided that assays of peritoneal fluid and lung would have little more to contribute, and they were not undertaken.

Weight changes

Ordinarily weanling rabbits show a measurable weight gain from day

to day. Daily weights were recorded for each animal except on days 5, 8 and 10. The significant determinations are shown in Fig. 13. Each symbol represents the change in weight in a single animal from the time of infection to the last weight taken before sacrifice. Animals are divided into 2 groups; those in which virus was recovered and those in which no virus was recovered. The data indicate that:

1. In the control group C-3, C-4 and C-5 and C-6 were able to gain at least a small amount of weight before sacrifice, despite their being severely ill and having virus in brain, liver and spleen. C-9 was free of virus and gained weight normally. C-11 was also free of virus, but it is possible that this animal was not infected intraperitoneally. It is not known why C-11 was unable to gain weight normally.
2. Three AzUR-treated animals yielded virus, but none lost weight. They were all clinically well. It might have been expected that AzUR-6 would have gained some weight. In this animal gross lesions of the liver and spleen were minimal, but the spleen was greatly enlarged. Of the 3 animals with no virus, AzUR-3 and AzUR-11 gained weight normally, but AzUR-9 gained, then later lost weight. This animal was found dead.
3. None of the IUdR-treated animals were able to gain weight. In the group of 3 with no virus, IUdR-9b showed considerable weight loss and was found dead. IUdR-9a had virus in his spleen and was also found dead.

4. In the ITSC-treated group, ITSC-3, ITSC-6, ITSC-9 and ITSC-11 gained weight normally and appeared clinically well. However, in the 2 animals in which virus was recovered, ITSC-5 gained no weight and ITSC-4 lost weight.

Discussion

Although intraperitoneal inoculation was used by Douglas, Smith and Price to produce severe vaccinia infections in rabbits, very little is known about the disease (73). The Levaditi strain of vaccinia does not cause a severe illness when inoculated intraperitoneally in mice (35). Ectromelia, however, is known to produce serious infections in mice after intraperitoneal inoculation (90, 184, 267). Fenner described this as an especially severe disease, with a rapid course of 5 days or less, involvement of the liver and spleen, and a characteristic accumulation of ascites and pleural effusions (90). Schell has stated that lethal infections result from intraperitoneal inoculation of only one particle of the highly infectious Moscow strain of ectromelia (267).

In this experiment a severe infection was produced in weanling rabbits following intraperitoneal inoculation with vaccinia. Adult rabbits are not as susceptible to the same infection (52, 174). The reasons for this difference are not known. In weanling rabbits the disease has a short incubation period before signs of clinical illness appear, usually by the third day. Animals become listless, anoretic and are soon moribund. At autopsy the liver and spleen show gross evidence of disease and there

is severe ascites. The disease pattern is similar to what has been described previously with intraperitoneal vaccinia in rabbits (73) and ectromelia (90). Although the course of clinical illness, morphologic findings and in some cases, viral titers are known for several intraperitoneal poxvirus infections, nothing is known of the physiologic disturbances which accompany severe clinical illness and lead to death. The specific role of antiviral chemotherapy can be evaluated only when all of the factors contributing to poxvirus toxicity are recognized. The findings of Boulter et al. are enough to suggest that antiviral chemotherapy alone is likely to be incomplete therapy in systemic poxvirus infections (34).

The systemic infection produced by intraperitoneal inoculation of weanling rabbits has been used to compare the antiviral activity of AzUR, IUdR and ITSC. During the acute period animals in each of the treated groups appeared clinically well, while untreated control animals were severely ill. Viral assays of brain, liver and spleen from all animals suggest that AzUR is moderately effective in suppressing vaccinia. IUdR and ITSC possess a greater degree of antiviral activity. The results with IUdR and AzUR in this systemic infection are similar to previous results in the therapy of cutaneous vaccinia in rabbits (49). In this milder infection IUdR was more effective than AzUR in suppressing viral lesions, but AzUR was definitely active. The present study has also shown that ITSC is effective as an antiviral agent in rabbits infected with vaccinia. This runs counter to the experience of Thompson

et al. who found thiosemicarbazones to have no effect against vaccinia in rabbits (247).

The clinical effects of therapy are reflected in the weight changes in each of the treated groups. Control animals rapidly became ill but did not lose weight. AzUR-treated animals followed a similar pattern except for AzUR-9. This animal was found dead but without virus. It is not known whether AzUR itself contributed to this outcome. IUdR-treated animals lost weight during the acute period whether or not they had virus and despite their appearance of being well. IUdR-9a and IUdR-9b also lost weight, and although one animal had virus, the fact that both were found dead after the acute period raises the question of drug toxicity. The dose of IUdR was 120mg/kg every 8 hours and was based upon previous studies in which the same dose was given to adult rabbits with no evidence of clinical toxicity (49). Weanling rabbits may be more susceptible to the toxic effects of IUdR than adult rabbits. Although 4 ITSC-treated rabbits with no virus gained weight, 2 animals had virus and did not gain weight. Both were sacrificed during the acute period. It is reasonable to assume that their lower weights reflect the infection rather than therapy, although there is no direct evidence to rule out drug toxicity. In this experiment, dose schedules for all three agents were based upon data derived from other systems. It was hoped that the question of drug toxicity would not have to be raised. The data, however, suggest it may have been a complication of therapy in some animals. Further studies in this system will be necessary to

determine a dose schedule for each agent which produces maximal antiviral activity with acceptable or no toxicity to the host.

Vaccinia is known to become resistant to IUdR (76, 166). Of all the animals sacrificed outside the acute period, virus was recovered from only one, IUdR-9b. Whether this was a resistant strain is not known. Future studies should attempt to demonstrate specific resistance to IUdR in any persisting virus, and also determine whether resistance is accompanied by any change in the virulence of the virus for the host.

Although AzUR, IUdR and ITSC have shown significant antiviral activity against vaccinia, therapy was begun at the time of infection, not at the onset of clinical disease. Cutaneous vaccinia in rabbits has been suppressed by IUdR when therapy was begun 48 hours after infection (49). In mice, ITSC may also confer protection against vaccinia when therapy is delayed. The results of the present experiment should encourage studies on the antiviral activity of AzUR, IUdR and ITSC when therapy is delayed until the onset of clinical disease.

In summary, a severe systemic infection has been produced in weanling rabbits by intraperitoneal inoculation of vaccinia virus. In this system, AzUR is moderately effective against vaccinia while IUdR and ITSC possess a greater degree of antiviral activity. Although questions have been raised regarding drug toxicity and the emergence of drug-resistant viruses, the demonstration of significant antiviral activity indicate that further studies with these agents in vaccinia infections may contribute to the search for an effective antiviral agent against clinical smallpox.

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Although [illegible], 7S and 19S were shown significant [illegible] against vaccinia, therapy was begun at the time of infection, not at the onset of clinical disease. Treatment initiated by [illegible] was administered by 1969 when therapy was begun 48 hours after infection with in mice, 1965 may also confer [illegible] against [illegible] until therapy is delayed. The results of the present experiment [illegible] studies on the antiviral activity of [illegible], 7S and 19S [illegible] delayed until the onset of clinical disease.

In summary, a severe systemic infection was [illegible] produced by intraperitoneal inoculation of vaccinia virus. [illegible] system, which is moderately effective against vaccinia, [illegible] 19S possess a greater degree of antiviral activity. Although [illegible] have been tested regarding their toxicity and the mechanism of their antiviral actions, the [illegible] of [illegible] indicate that further studies with these agents [illegible] are warranted [illegible] potential [illegible] clinical usefulness.

V. FINAL CONSIDERATIONS ON ANTIVIRAL CHEMOTHERAPY

The search for effective chemotherapeutic agents for the treatment of viral diseases is in its infancy. By comparison, the development of anti-bacterial agents has been enormously successful. The goal has been to discover the fundamental biochemical mechanisms of the offending microorganism or virus which are qualitatively different from those of the host cell and vulnerable to attack by chemotherapeutic agents. Despite the success of antimicrobial agents, these mechanisms have been elucidated in only a few instances.

With viruses the task is far more difficult. Their intracellular habitat makes them elusive. The similarity of their biochemical mechanisms to those of the host cell make the search for subtle qualitative differences far more formidable. In some instances, the hope is merely to find an essential biochemical property of the virus which is quantitatively different from that of the host cell.

Yet for all of these difficulties progress is being made. With each advance in the understanding of the molecular biology of viruses, new sites of attack appear. The enormous efforts that have gone into the search for anti-neoplastic agents has already uncovered compounds with exciting antiviral possibilities.

The largest proportion of viral infections that affect man are mild, transient, often asymptomatic and more a nuisance than a threat to human life. Many people would be willing to accept as part of the vissicitudes

of life the social and economic loss which result from repeated viral illnesses. But not all viral infections are inocuous. A few viruses are always virulent, and many of the so-called inocuous viruses may cause severe complications and often death in those who suffer from totally unrelated diseases. Efforts to contain viral diseases have been directed toward programs of prevention--sanitation and vector control, vaccination and quarantine. Success in these areas has been enormous, but by no means universal. Wherever poverty and ignorance are found, success will always be partial . Where indifference appears, disease will strike. And once it has struck it must be treated.

Few virus diseases better illustrate this problem than smallpox. Despite widespread vaccination, large populations still go unprotected. In regions where vaccination has made smallpox an unknown disease, immunity wanes and the danger of widespread epidemic is ever present. Each year there are 100,000 cases of smallpox and 25,000 deaths from this disease. The toll in human life in many areas of the world is frightening; the threat even to the most civilized communities an outrage. There exists a real need for an effective agent in the treatment of smallpox and it is hard to imagine a time when preventive measures will become so successful that such an agent is unnecessary.

Recent studies in experimental poxvirus infections have shown that the thiosemicarbazones, 5-iodo-2'-deoxyuridine and 6-azauridine possess significant antiviral activity. Experience thus far should encourage further investigation of these agents as part of the continuing search for an effective agent against clinical smallpox.

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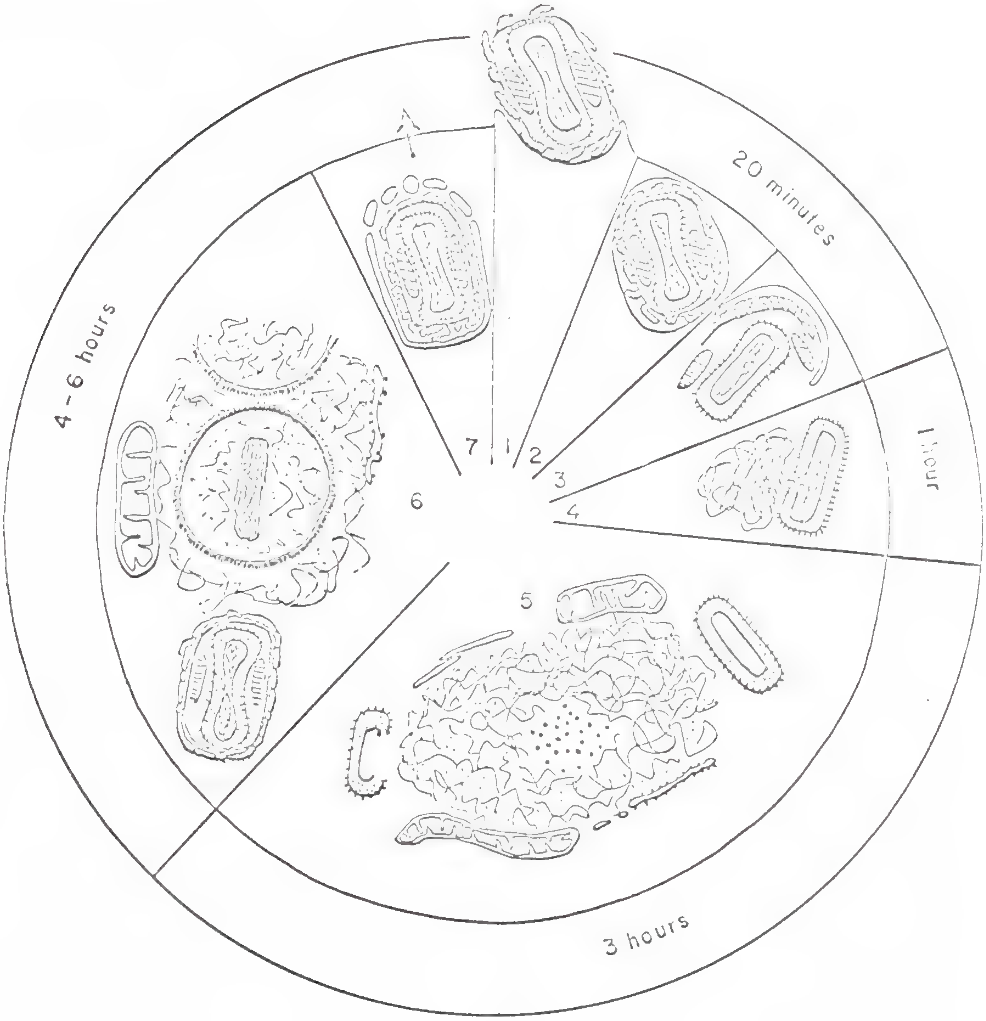
VII. FIGURES

The Sequence of Vaccinia Uptake and Replication in L Cells

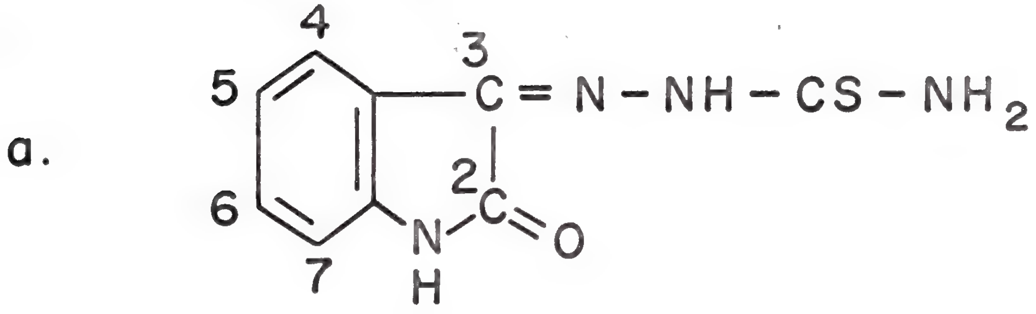


Within a few minutes of infection, vaccinia is adsorbed to the outer membrane of the cell (1). It is then transferred by phagocytic vesicle into the cell (2) where its outer coat together with the vesicle is disrupted releasing the DNA-containing viral core into the cytoplasm (3). Viral cores reach their maximum number one hour after infection. By this time, viral cores begin to break down, releasing their DNA (4). Sometimes the core breaks down completely; at other times a break appears in its surface allowing the DNA to leak out. This "naked DNA" then comes together in foci of viroplasm or "factories" which are surrounded by clusters of ribosomes (5). It is here that new virus particles are synthesized. Three hours have elapsed since infection.

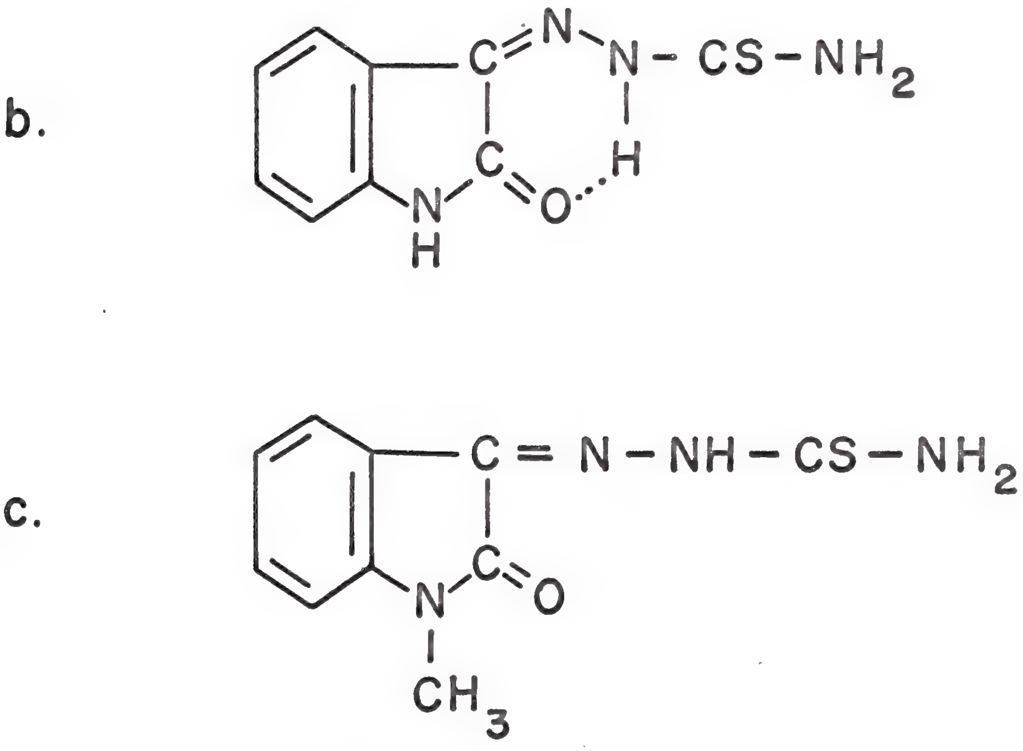
At four hours spherical membranes coated on their outer side by short cylindrical projections become evident within the "factories" (6). A large proportion of these immature virus particles possess dense nucleoids. Occasionally nucleoids without surrounding membranes are observed. Each nucleoid consists of dense filaments arranged parallel to the long axis of the nucleoid. Viral material once segregated within a surrounding membrane undergoes rearrangement into the brick-shaped mature infectious viral particle (7). (Adapted from Dales S. J. Cell Biol., 18:51-72, 1963.)

Fig. 1

STRUCTURE OF ISATIN-β-THIOSEMICARBAZONE



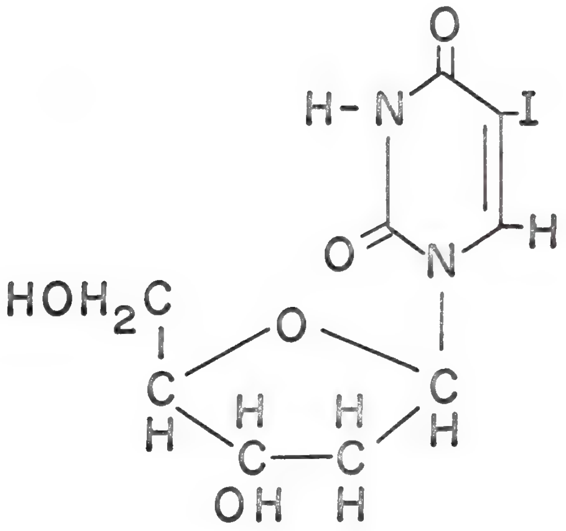
ISATIN-β-THIOSEMICARBAZONE



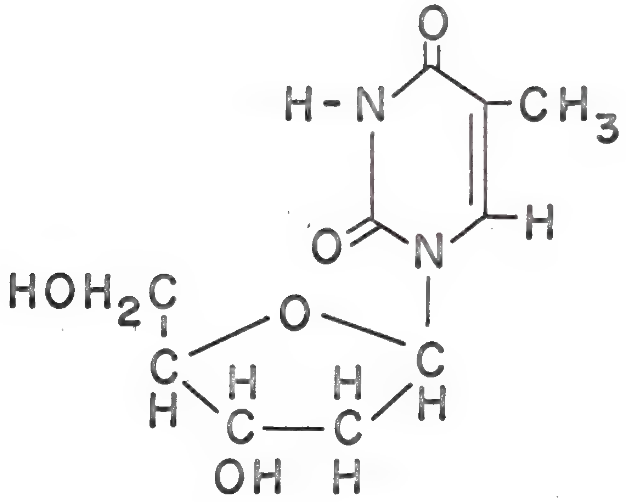
N-METHYL-
ISATIN-β-THIOSEMICARBAZONE

FIG. 2

STRUCTURAL SIMILARITY BETWEEN IUdR AND THYMIDINE



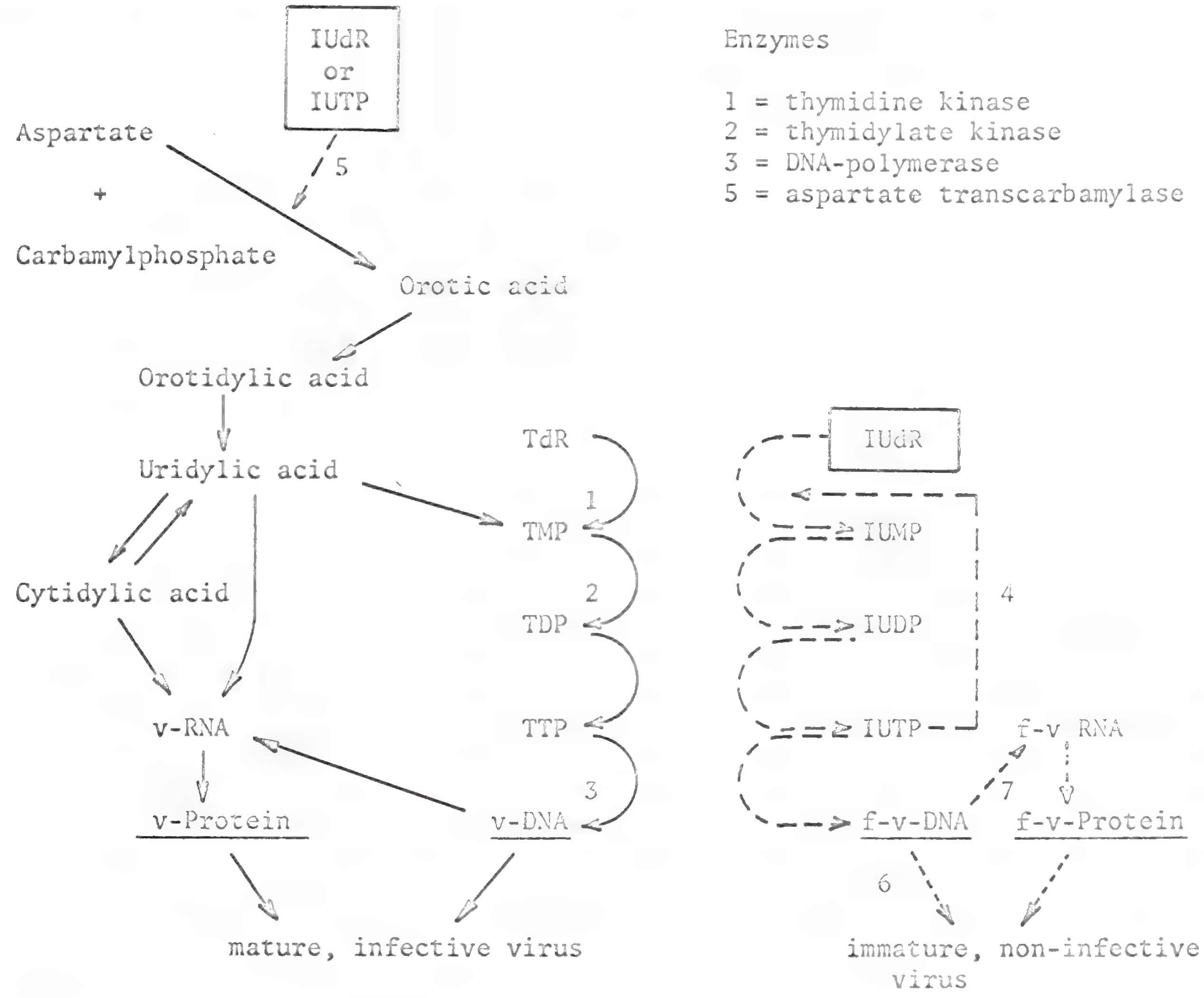
5-IODO-2'-DEOXYURIDINE (IUdR)



THYMIDINE (TdR)

FIG. 3

Possible Sites of Antiviral Activity of 5-iodo-2'-deoxyuridine (IUdR)

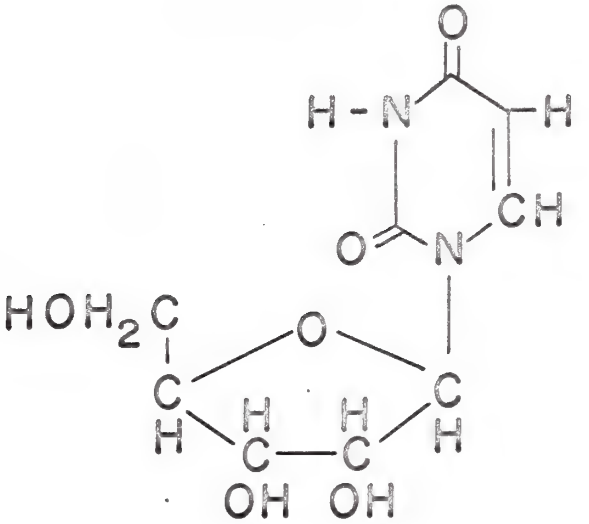


Legend:

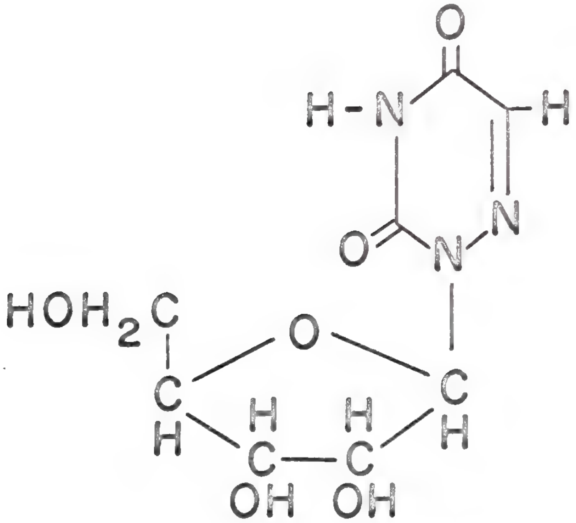
TdR = thymidine; TMP, TDP and TTP = thymidine mono-, di-, and tri-phosphate
IUdR = 5-iodo-2'-deoxyuridine; IUMP, IUDP and IUTP = IUdR mono-, di-, and tri-phosphate
v = viral f-v = fraudulent viral

Fig. 4

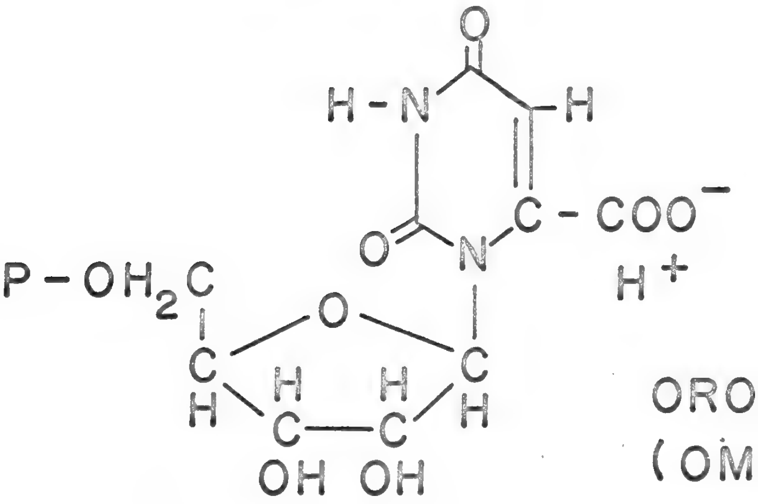
STRUCTURAL SIMILARITIES BETWEEN URIDINE, 6-AZAURIDINE AND OROTIDYLIC ACID



URIDINE (UdR)



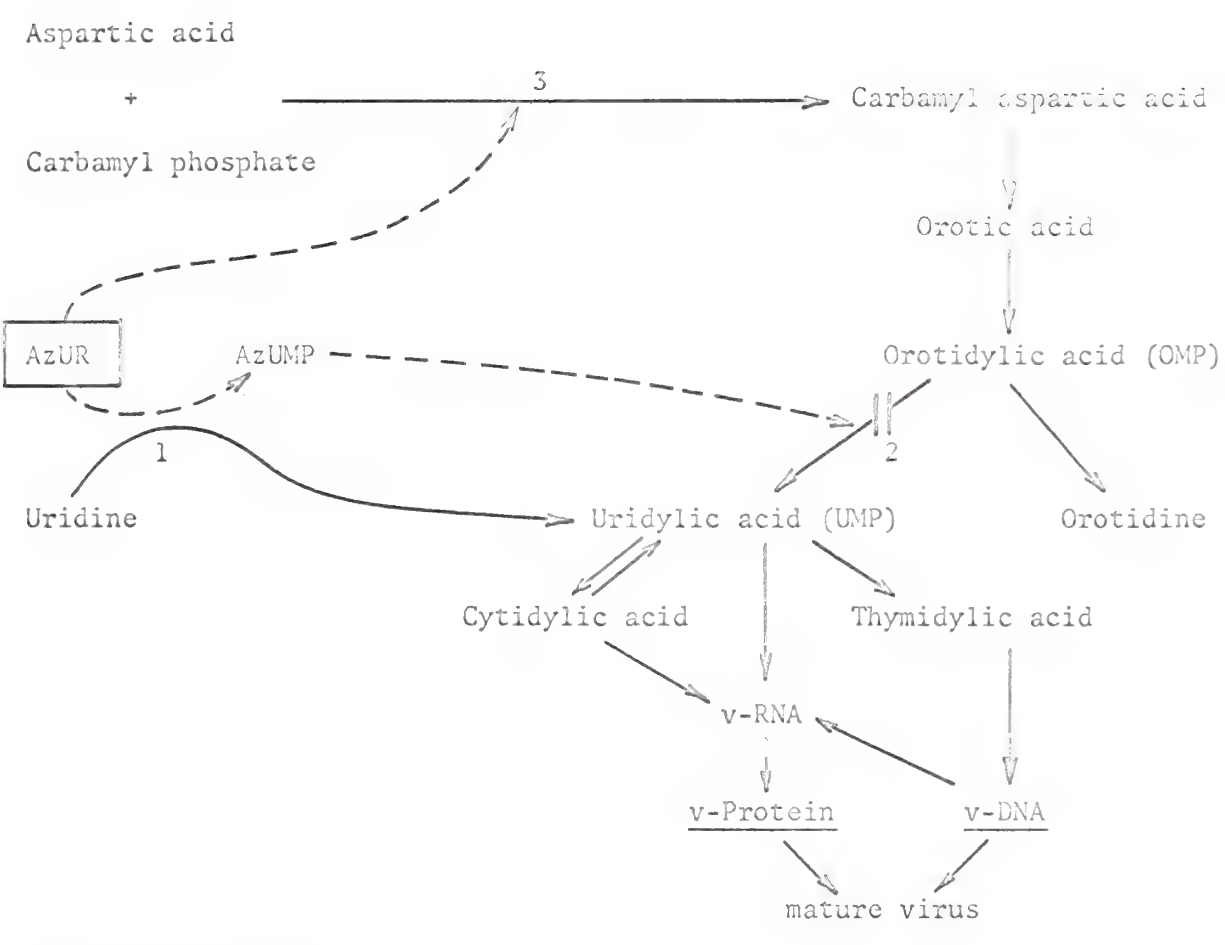
6-AZAURIDINE
(AzUR)



OROTIDYLIC ACID
(OMP)

FIG. 5

Possible Sites of Antiviral Activity of 6-Azauridine (AzUR)



1 = Uridine kinase
2 = Orotidylic acid decarboxylase
3 = Aspartate transcarbamylase

Fig. 6

POSSIBLE SITES OF ANTIVIRAL ACTION OF AzUR, IUdR, AND ITSC

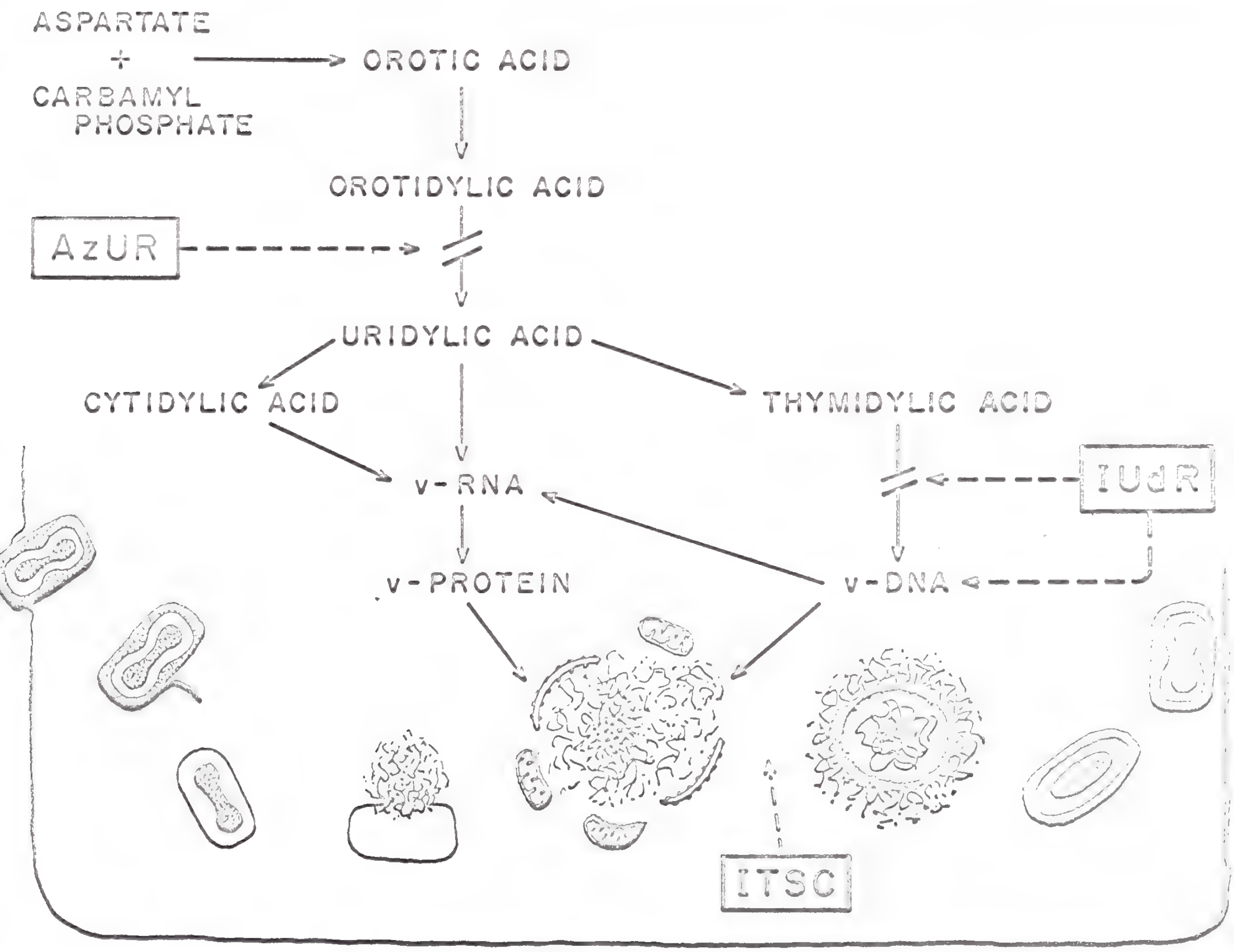


FIG. 7

COMPARISON OF THREE ANTIVIRAL AGENTS USED IN POX VIRUS INFECTIONS *IN VIVO*

VIRUS	HOST	SITE OF INFECTION	AzUR	IUdR	ITSC
VACCINIA	MOUSE	INTRACEREBRAL	?	?	++++
	RABBIT	CORNEAL	0	+++	?
		INTRADERMAL	++	++++	+
		INTRACEREBRAL	?	?	0
		SYSTEMIC (INTRAPERITONEAL)	?	?	?
	MAN	INTRADERMAL	0	++++	?
		GENERALIZED	?	?	±
VARIOLA MAJOR	MAN	PROPHYLAXIS	?	?	+++
		CLINICAL DISEASE	?	?	0

FIG. 8

Gross Lesions Seen at Autopsy

Ascites	Control		++++	++++	++++	±	0
	AzUR	0	±	++	±		±
	IUdR	0	0	0	±	0,0	
	ITSC	±	0	±		±	±
Spleen	Control		+++	+++	++	+	0
	AzUR	0	0	0	0	0	0
	IUdR	0	0	0	0	0,0	
	ITSC	0	0	0		0	±
Liver	Control		+	+	++	+	0
	AzUR	0	0	0	±	0	0
	IUdR	0	0	0	0	±,0	
	ITSC	±	0	0		+++	±
		3	4	5	6	9	11

DAY

Fig. 9

VIRUS TITERS IN BRAINS OF CONTROL AND TREATED RABBITS INFECTED WITH VACCINIA

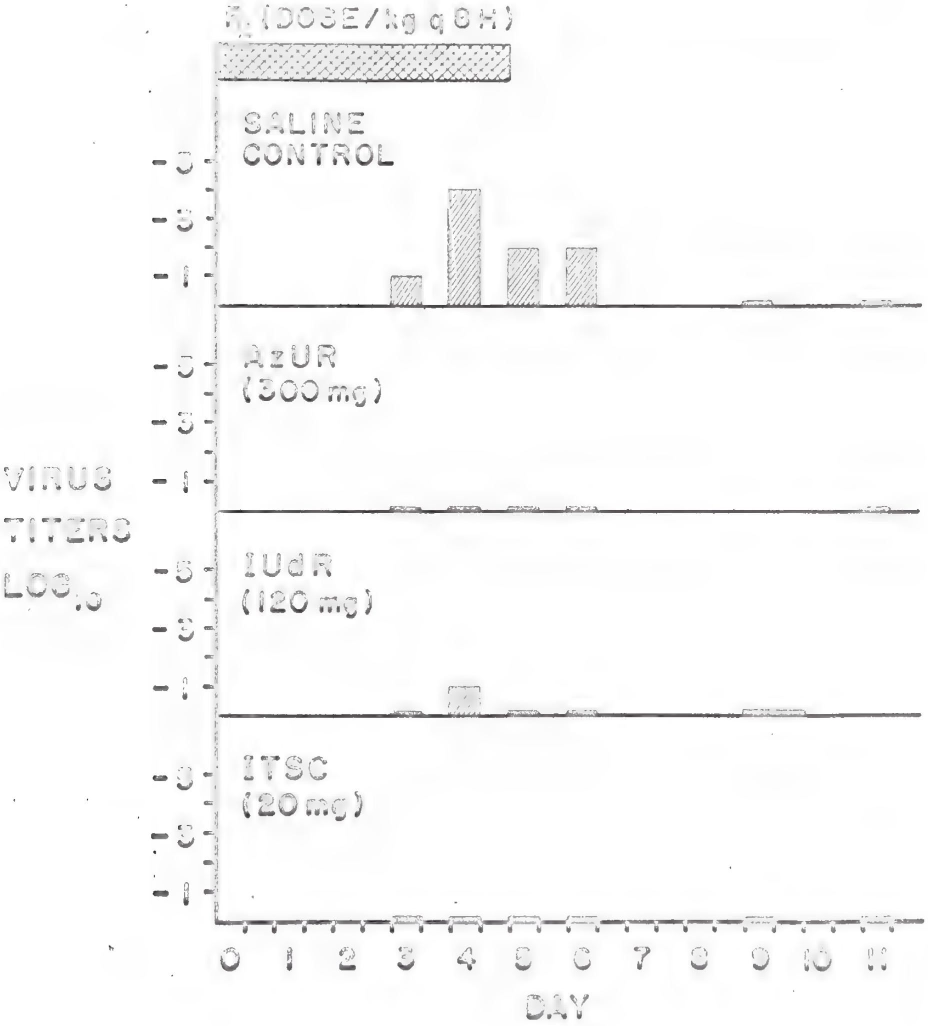


FIG. 10

VIRUS TITERS IN LIVERS OF CONTROL AND TREATED RABBITS INFECTED WITH VACCINIA

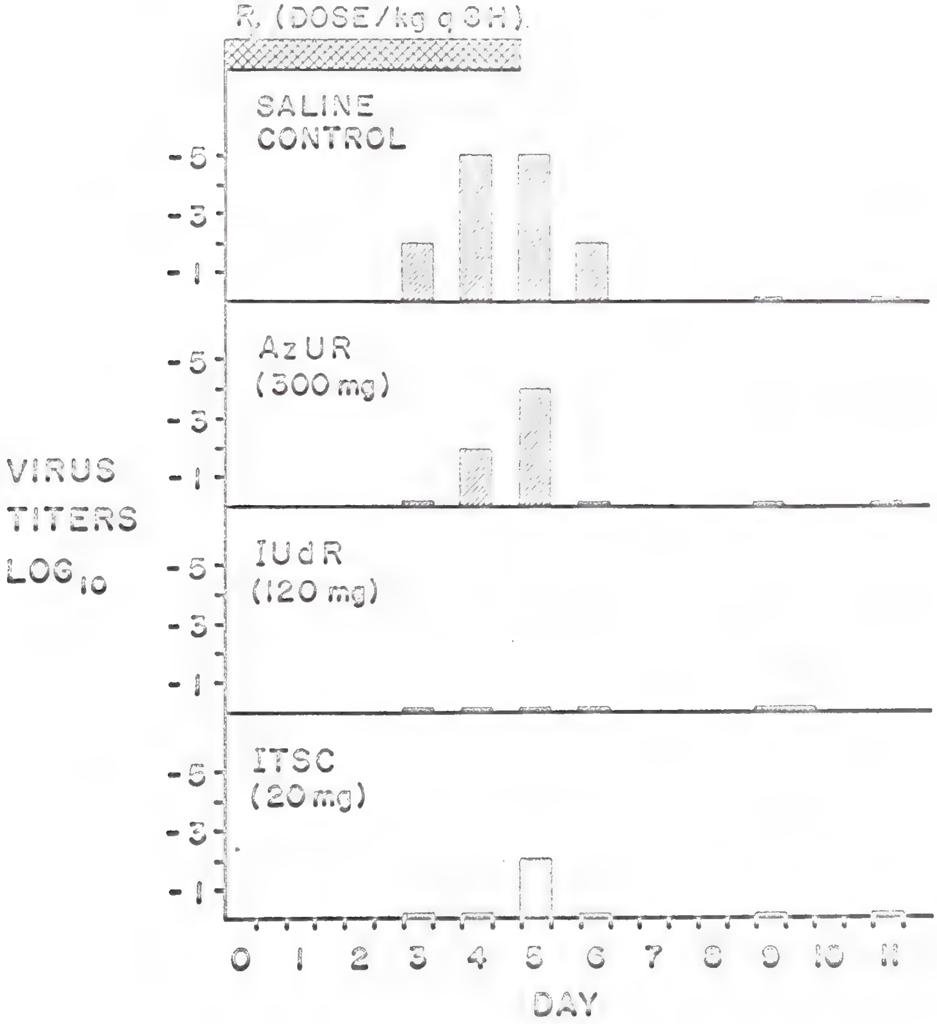


FIG. 11

VIRUS TITERS IN SPLEENS OF CONTROL AND TREATED RABBITS INFECTED WITH VACCINIA

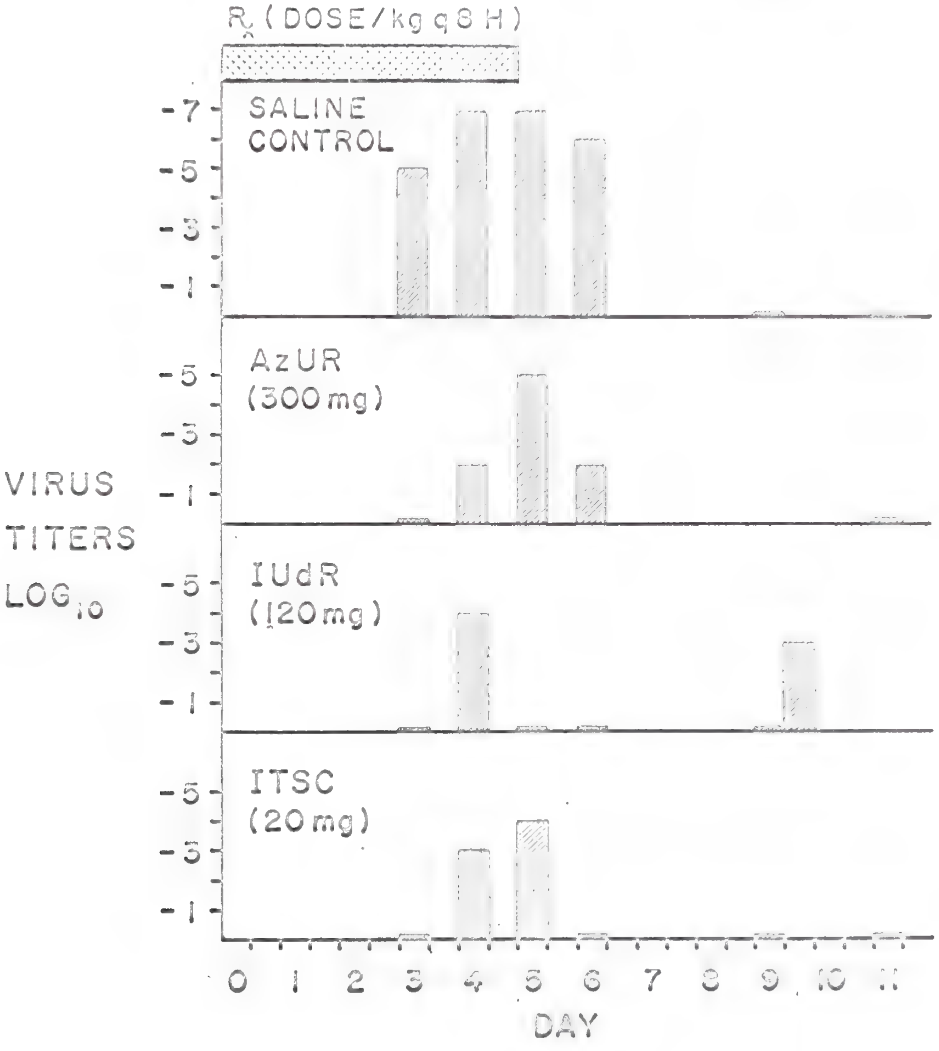


FIG. 12

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